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**THE EFFECT OF 5,6-DIMETHYLBENZIMIDAZOLE ON VITAMIN B12
SYNTHESIS AND PROTEIN PRODUCTION
IN *PROPIONIBACTERIUM FREUDENREICHII* SSP. *FREUDENREICHII***

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Tiivistelmä/Referat – Abstract <p><i>Propionibacterium freudenreichii</i> is commonly known as a Swiss-type cheese ripening culture, and is also frequently applied in the industrial production of vitamin B12 (cobalamin). One way to increase vitamin B12 productivity is the supplementation of medium with 5,6-dimethylbenzimidazole (DMBI), the lower ligand of cobalamin. The aim of this thesis was to investigate the effect of exogenous DMBI on vitamin B12 synthesis and protein production in type strain of <i>P. freudenreichii</i> ssp. <i>freudenreichii</i>.</p> <p>Results showed that DMBI supplementation improved cobalamin production 2.7- and 2.0-fold in cultures grown in anaerobic and two-step processes, respectively. Two-step incubation enabled the achievement of significantly higher levels of cobalamin both in the absence and presence of DMBI. The highest cobalamin level of 0.6 µg/ml was achieved by the combination of DMBI supplementation with the two-step process.</p> <p>Proteome maps displayed 474 protein spots which is less than 20% of <i>P. freudenreichii</i> predicted proteome. No significant differences in protein production were found between cultures grown in the absence and presence of DMBI. Only two protein spots were more abundant in presence of DMBI and four spots showed higher abundance in absence of DMBI. None of detected proteins from these spots were found to be directly involved in cobalamin biosynthesis.</p>			
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PREFACE

This study was carried out at the Department of Food and Environmental Sciences, Faculty of Agriculture and Forestry, University of Helsinki.

I would like to express my gratitude to my supervisor Pekka Varmanen for advice and guidance. I am very grateful to Kirsi Savijoki who was helping and advising me in all issues. I would also like to thank Paulina Deptula, PhD student, who helped me to learn 2D gel electrophoresis and shared her experience which was of great value for me.

It was a great pleasure to work together with my fellow EMFOL student and friend Maria Asuncion Fernandez Lopez.

Helsinki, August 2014

Tatiana Ishchenko

LIST OF ABBREVIATIONS

2D GE	two-dimensional gel electrophoresis
ACNQ	2-amino-3-carboxy-1,4-naphthoquinone
ALA	5-aminolaevulinic acid
ATR	acid tolerance response
BCCP	biotin carboxyl carrier protein
BGS	bifidogenic growth stimulator
BSA	bovine serum albumin
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
CLA	conjugated linoleic acid
DHNA	1,4-dihydroxy-2-naphthoic acid
DMBI	5,6-dimethylbenzimidazole
DTT	dithiothreitol
EFSA	European food safety authority
FDA	Food and Drug Administration agency
FFA	free fatty acids
FMN	flavin mononucleotide
FMNH ₂	reduced flavin mononucleotide
GRAS	generally recognized as safe
IEF	isoelectric focusing
IPG	immobilized pH gradient
LC-MS/MS	liquid chromatography tandem mass spectrometry
OD	optical density
PBS	phosphate buffered saline
PCA	principal component analysis
PPA	propionic agar
QPS	qualified presumption of safety
TCA	tricarboxylic acid cycle
TFA	trifluoroacetic acid

TGS Tris Glycine Sodium dodecyl sulfate buffer

SCFA short-chain fatty acid

SDS-PAGE sodium dodecyl sulfate polyacrylamide gel electrophoresis

UHPLC ultra high pressure liquid chromatography

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1. INTRODUCTION

Propionibacterium freudenreichii is used as a ripening culture in Swiss type cheeses. It metabolizes lactate and produces carbon dioxide which results in eye formation in cheese body (Thierry et al. 2010). *P. freudenreichii* also contributes greatly to flavor development in cheese (Thierry et al. 2011). Based on long tradition of use, *P. freudenreichii* has been granted both GRAS (generally regarded as safe) and QPS (qualified presumption of safety) statuses in the USA and EU, respectively (Mogensen et al. 2002; EFSA 2008).

Propionic acid bacteria have also emerged as probiotic candidates. They appear to adapt well in the gastrointestinal environment. They are known to produce bifidogenic compounds and exhibit broad range of other beneficial activities, including antimicrobial and antimutagenic properties (Cousin et al. 2011). Propionic acid bacteria are known to produce a range of bioactive compounds, including vitamins (cobalamin, folate, riboflavin) (Poonam et al. 2012), conjugated linoleic acid (CLA) (Andrade et al. 2012) and short chain fatty acids (Jan et al. 2002).

P. freudenreichii is also used in industrial production of vitamin B12 (cobalamin) (Martens et al. 2002). It utilizes anaerobic B12 biosynthetic pathway which includes about 30 enzyme-driven reactions (Raux et al. 2000). The pathway has been elucidated fully except for biosynthesis of cobalamin lower ligand – 5,6-dimethylbenzimidazole (DMBI). DMBI as a lower ligand is highly important for cobalamin biological activity. In humans, only two enzymes are cobalamin dependent: methionine synthase and methylmalonyl-CoA transferase (Martens et al. 2002). If DMBI is replaced with other bases, B12-like compound becomes inactive for humans, e.g. pseudocobalamin containing adenine in the lower ligand position (Vorobjeva 1999; Hazra et al. 2013).

In industrial process of vitamin B12 production which is conducted anaerobically, DMBI is added to the growth medium which allows achieving high levels of cobalamin production (Wang et al. 2012). Alternatively, the process is conducted in two steps including anaerobic incubation for a few days followed by switch to aeration mode. With oxygen supply provided, the biosynthesis of own bacterial DMBI is stimulated (Martens et al. 2002).

P. freudenreichii produces DMBI in oxygen dependent pathway from riboflavin (Renz and Weyhenmeyer 1972) . This pathway is not well understood yet. It is known to be catalyzed by BluB enzyme which belongs to ‘flavin destructase’ family (Taga et al. 2007). The enzyme was found in both in anaerobic (*Bacillus megaterium*) (Gray and Escalante-Semerena 2007) and aerobic (*Sinorhizobium meliloti*) (Campbell et al. 2006) producers of vitamin B12.

In this work a proteomic approach was used to explore the effect of DMBI on global protein production by type strain of *P. freudenreichii*. Proteomics is a common tool for investigation of physiological responses to different stimuli in bacterial cells (Han et al. 2011). Gel-based proteomic technique was previously applied in *P. freudenreichii* to study the mechanisms of bile (Leverrier et al. 2003), heat (Anastasiou et al. 2006) and acid adaptation (Jan et al. 2001).

The aim of this thesis was to study the effect of exogenous DMBI in growth medium on vitamin B12 synthesis in type strain of *P. freudenreichii* ssp. *freudenreichii*. Vitamin B12 production was evaluated in two types of processes (fully anaerobic and two step system). The second constituent of the study was the investigation of protein production in *P. freudenreichii*. Proteome maps were obtained at the mid-logarithmic growth stage for the cultures grown in presence and absence of DMBI. They were used to detect and identify proteins expressed differentially between two conditions.

The literature review introduces general features of *P. freudenreichii* species and its metabolism. The role of propionic acid bacteria in cheese is discussed as well as the potential to be used in other fermented products. Different aspects of probiotic potential of propionic acid bacteria are introduced including data from *in vitro* and *in vivo* studies. Vitamin B12 biosynthesis is outlined with special place dedicated to lower ligand structure. Biosynthesis of DMBI from riboflavin via aerobic pathway is described in details. The strategies of enhancing cobalamin productivity in industrial applications are discussed. Finally, proteomics as a tool to study bacterial cell metabolism is introduced including the overview of strengths and disadvantages. An insight into proteomic data available for *P. freudenreichii* is made.

2. LITERATURE REVIEW

2.1 Overview of *P. freudenreichii*

2.1.1 General characteristics of *P. freudenreichii* species

Genus *Propionibacterium* belongs to Actinobacteria class, family Propionic acid bacteriaeae. They are Gram-positive, rod shaped, non-motile, non-sporeforming, generally catalase positive bacteria characterized by high G+C content (64-67 mol%). Phylogenetic distinction between species is based on 16S rRNA gene sequence. Propionic acid bacteria are mainly anaerobic to aerotolerant and may be microaerophilic (Whitman et al. 2012).

The genus *Propionibacterium* includes 12 species divided into two groups based on their habitat (Cousin et al. 2011):

- 1) Dairy propionic acid bacteria: *P. acidipropionici*, *P. cyclohexanicum*, *P. freudenreichii*, *P. jensenii*, *P. microaerophilum*, *P. thoenii*
- 2) Cutaneous propionic acid bacteria: *P. acidifaciens*, *P. acnes*, *P. australiense*, *P. avidum*, *P. granulosum*, *P. propionicum*.

Dairy (or classic) propionic acid bacteria can be found in other than milk-derived foods including fruits and vegetables (Mantere-Alhonen 1995; Vorobjeva et al. 2008). In addition, they are also found in soil and silage (Whitman et al. 2012).

P. freudenreichii is a type species of the genus. It was named after Swiss bacteriologist Eduard von Freudenreich who first isolated and described the species. In 1906 in co-authorship with Orla-Jensen he showed relation of eyes formation in cheese with propionic acid producing bacteria. The first genome of *P. freudenreichii* strain CIRM-BIA1^T (CIP103027) was sequenced a few years ago by Falentin et al. (2010).

P. freudenreichii species includes nowadays two subspecies: *freudenreichii* and *shermanii*. They are traditionally distinguished by two features: nitrate reduction and lactose fermentation. *P. freudenreichii* ssp. *freudenreichii* is able to reduce nitrate and lacks the ability to ferment lactose. By contrast, *P. freudenreichii* ssp. *shermanii* metabolizes lactose but lacks nitrate reduction (Whitman et al. 2012).

2.1.2 Metabolism of *P. freudenreichii*

Propionic acid bacteria have low nutritional requirements and are able to survive in very unfavourable conditions. They may grow without the addition of amino acids (Whitman et al. 2012). The genome sequence defined for *P. freudenreichii* strain CIRM-BIA1^T was found to encode pathways for synthesis of all vitamins excluding those for pantothenate and biotin (Falentin et al. 2010). Accordingly, all strains examined to date require only pantothenate and biotin for growth (Whitman et al. 2012). Propionic acid bacteria can catabolize a variety of carbon sources, including sugars (D-glucose, D-mannose, rhamnose, galactose, lactose, sucrose, trehalose), lactate and alcohols (glycerol, mannitol, adonitol, erythriol) (Whitman et al. 2012).

The genus *Propionibacterium* is generally characterized by high production of propionic acid. Propionate is produced by a pathway named Wood-Werkman or transcarboxylase cycle (Fig. 1). The pathway is characterized by high ATP yield. In propionic acid bacteria it is the main way of energy generation (Vorobjeva 1999).

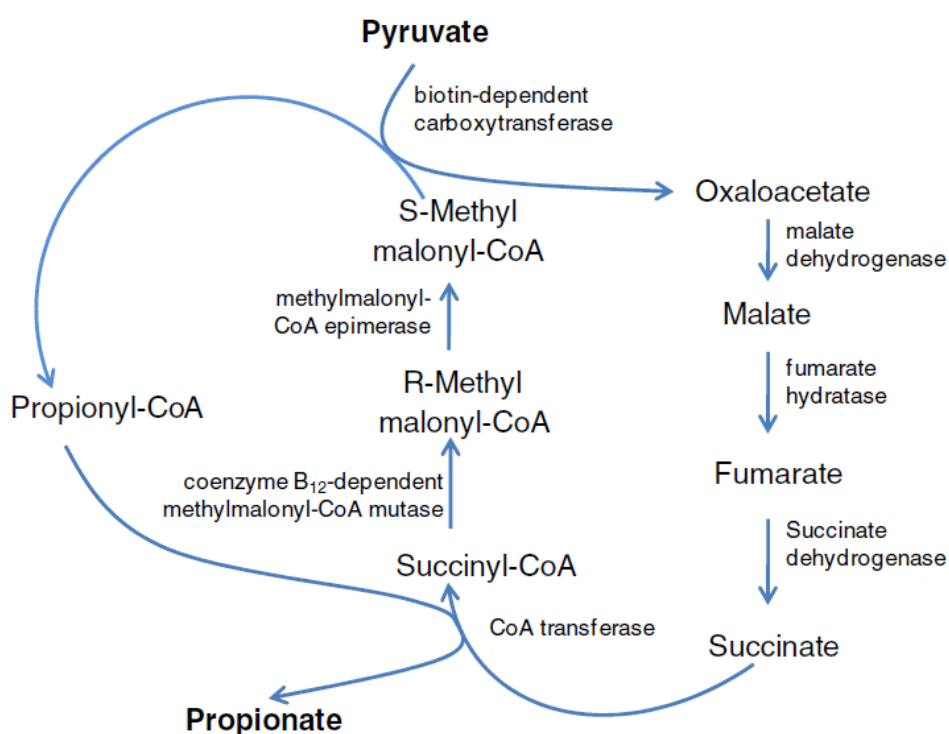


Figure 1. Wood-Werkman cycle in *P. freudenreichii* (adapted from Thierry et al. 2011)

The essence of Wood-Werkman cycle is the reduction of pyruvate to propionate. One of the key reactions is transcarboxylation between methylmalonyl-CoA and pyruvate yielding oxaloacetate and propionyl-CoA. This reaction is catabolized by biotin-dependent methylmalonyl-CoA carboxytransferase without energy consumption. Oxaloacetate then enters tricarboxylic acid cycle (TCA cycle) where it is converted to succinate. As a result of reduction of fumarate to succinate ATP is generated. Propionyl-CoA exchanges coenzyme A group with succinate yielding propionate which is mediated by CoA transferase. Finally, methylmalonyl-CoA mutase converts succinyl-CoA to methylmalonyl-CoA. The enzyme uses adenosylcobalamin (vitamin B12) as a cofactor. (Thierry et al. 2011).

In order to maintain redox potential, part of pyruvate is oxidized to acetate and carbon dioxide (Thierry et al. 2011). The ratio propionic:acetic acid is typically 2:1, but may reach 5:1 or higher (Dworkin et al. 2006). Genome sequencing by Falentin et al. (2010) reconstituted both Wood-Werkman pathway and oxidative decarboxylation pathways.

Although *P. freudenreichii* are predominantly anaerobic, they possess necessary genes for respiration in aerobic condition such as NADH dehydrogenase, succinate dehydrogenase, cytochrome *bd* complex, ATPase and the complete pathway for heme synthesis (Falentin et al. 2010). The response to oxygen is known to be strain-dependent (Vorobjeva 1999).

Some strains of *P. freudenreichii* possess aspartase activity. Aspartate is metabolized to succinate (via fumarate) and ammonia. This affects lactate fermentation by re-directing to CO₂ and acetate formation (Crow 1986). Excessive aspartase activity in strains used in cheese-making causes the risk of late fermentation which may lead to split defects. Aspartase activity is therefore considered when selecting strains for cheese-making (Blasco et al. 2011).

2.1.3 Role of propionic acid bacteria in cheese ripening

The primary use of propionic acid bacteria in food fermentations is ripening cultures for Swiss-type cheeses (Thierry et al. 2010). This is partly due to its comparatively high thermotolerance, enabling this starter culture to reach levels as high as 10⁹ CFU/g (Thierry et al. 2011). The most well-known variety of Swiss type cheeses is Emmental. The characteristic step in technology of Emmental includes the step of cooking at 50-60°C for

about 30 min which constitutes stress for cheese microflora. The ripening is accomplished at warm temperature 18-24°C for at least 4-6 weeks (Thierry et al. 2010).

During ripening in Swiss type cheeses propionic acid bacteria anaerobically utilize lactate derived from lactose fermentation performed by lactic acid bacteria, yielding propionate, acetate and carbon dioxide. Accumulation of carbon dioxide results in formation openings (eyes) in cheese body.

Propionic acid bacteria contribute greatly also to cheese flavor formation during ripening. They produce a wide range of non-volatile and volatile compounds (Thierry et al. 2010). Volatile compounds result from the catabolism of branched-chain, aromatic, and sulfur-containing amino acids. Propionic acid bacteria were shown to significantly influence the amount of 57 out of the 69 volatiles in cheese juice (Thierry et al. 2004). In small scale Swiss cheeses production propionic acid bacteria increased levels of all carboxylic acids and 14 out of 58 neutral flavor compounds as compared to cheese ripened without them (Thierry et al. 2005). Being added as adjunct culture to traditional Raclette cheese it significantly affected the level of 16 volatile compounds and increased the level of free fatty acids two- to five-fold. Cheese then received higher scores for odor and flavor intensity in sensory evaluation (Thierry et al. 2005).

One of the most important branched-chain flavor compounds produced by propionic acid bacteria in cheese are 3-methylbutyric acid and 2-methylbutyric acid. The former is referred to as isovaleric acid. These acids are derived from leucine and isoleucine catabolism, respectively (Thierry et al. 2004). Isoleucine was found to be converted more efficiently than leucine resulting in prevalence of 2-methylbutyric acid over 3-methylbutyric acid (approximately 80:20) (Thierry et al. 2011). As it was reviewed by Thierry et al. (2002), these acids along with isobutyric and isocaproic acids render the cheese sweaty, fruity, sweet, cheesy and fatty acid-like flavor (Thierry and Maillard 2002). The levels of methylbutyric acids were 3 to 10 times higher (depending on strain) in presence of propionic acid bacteria in the starter culture although they do not appear to be the only producers (Thierry et al. 2004). Leucine and isoleucine catabolism starts with transamination yielding in α -ketoacid followed by its decarboxylation and oxidation in one or several steps to form final volatile acid (Thierry et al. 2004; Thierry et al. 2011).

Propionic acid bacteria also significantly affect lipolysis of milk fat and free fatty acids (FFA) release although this activity is strain specific. FFAs impart cheese pungent, rancid,

cheesy and fruity notes (Collins et al. 2003). In presence of propionic acid bacteria FFA levels were two to eight times higher than in their absence. In recent work, it was shown that the secreted esterase encoded by the *P. freudenreichii* CIRM-BIA1^T gene *pf279* is the main lipolytic enzyme involved in Swiss cheese ripening process. Inactivation of the *pf279* gene reduced the lipolytic activity by 75% compared to that of the wild-type strain (Abeijon Mukdsi et al. 2014).

Formation of esters in cheese is also significantly affected by propionic acid bacteria. Both the variety of esters and their level are improved. Some esters can be found only at presence of propionic acid bacteria, mainly those composed of propionate and acetate that are supplied from lactate fermentation. Propionic acid bacteria are likely to contribute also to ester synthesis (Thierry et al. 2004).

Flavor in cheese is known to develop during ripening at warm temperature and continue during cold storage. This is to high extent due to the activity of propionic acid bacteria maintained at cold temperatures. It was shown that 20% of propionate and acetate, ~25% of short chain FFA, ~41% of long-chain FFA, ~60% of branched-chain acids from isoleucine and leucine catabolism, and ~80% of esters are formed during cold storage of cheese (Thierry et al. 2005).

2.1.4 Propionic acid bacteria as probiotics

2.1.4.1 Safety and adaptation in GIT

Probiotic is defined as “a live microorganism which, when administered in adequate amounts, confers a health benefit on the host” (FAO 2006). Dairy propionic acid bacteria emerged as probiotic candidates and that has been shown in numerous studies *in vitro*, *ex vivo*, *in vivo* in animal models and humans (Cousin et al. 2011). The first evidence of propionic acid bacteria as monoculture delivering health benefits were shown in animal study involving piglets. The administration of *P. freudenreichii* with fodder resulted in better weight gain, lowered feed demand and less diarrhea in piglets (Mantere-Alhonen 1995).

Dairy propionic acid bacteria are recognized to be safe for humans. For instance, in a study with *P. freudenreichii* ssp. *shermanii* that was given in a mixture with other three probiotics to infants for six months, no risks for health were registered (Kukkonen et al. 2008). Based on the long term application in cheese making they acquired both ‘Generally

recognized as safe' (GRAS) from American Food and Drug Administration (FDA) (Mogensen et al. 2002) and 'Qualified presumption of safety' (QPS) status from the European food safety authority (EFSA) (EFSA 2008).

Propionic acid bacteria express tolerance to digestive tract environment including acidity and bile salts. Bile salts are animal detergents that are highly toxic for microorganisms (Leverrier et al. 2003). Acid and bile tolerance appear to be a strain dependent feature. Many strains can survive pH values as low as 4.0 (Yuksekdag et al. 2014). And there are strains with excellent survival rate at pH 3.0 (Suomalainen et al. 2008) It is noteworthy that probiotics delivered with certain vectors like fermented foods express higher tolerance to digestive stress than pure cultures (Cousin et al. 2011). This was shown, for instance, during *in vitro* studies with fermented milk (Leverrier et al. 2005). Detection of viable cells after passage through GIT was reported in a few *in vivo* studies; high numbers of *P. freudenreichii* could be found in feces of human microbiota-associated rats (Lan et al. 2007). Viable cells of *P. freudenreichii* ssp. *shermanii* JS strain have also been detected in feces of adult volunteers (Suomalainen et al. 2008).

The number of dairy propionic acid bacteria in human gut is low but their colonization levels can be increased through oral administration (Vorobjeva et al. 2008). Propionic acid bacteria are able to adhere to the intestinal cells, which was shown both *in vitro* and in animal model (Cousin et al. 2011). The studies involving human intestinal mucosa indicated that propionic acid bacteria displayed comparatively low adhesion efficacy (Thiel et al. 2004). *P. freudenreichii* ssp. *shermanii* strain was shown to be able to adhere both to human and mice intestinal epithelial cells, and porcine mucus (Zárate et al. 2002; Yeruva et al. 2011). In a recent study involving piglets, *P. freudenreichii* strain demonstrated adaptability and physiological activity in the colon environment (Saraoui et al. 2013).

2.1.4.2 Bifidogenic properties

Propionic acid bacteria can promote growth of well-established probiotic microorganisms such as *Bifidobacterium*. They are known to produce two bifidogenic growth stimulators (BGS): 1,4-dihydroxy-2-naphthoic acid (DHNA) and 2-amino-3-carboxy-1,4-naphthoquinone (ACNQ) (Cousin et al. 2011). DHNA is a precursor of vitamin K (menaquinone), it also stimulates intestinal bifidobacteria and ACQN may be derived from DHNA (Isawa et al. 2002, Cousin et al. 2011). ACQN as a growth stimulator of

Bifidobacterium was discovered in cell-free extract of *P. freudenreichii* (Yamazaki et al. 1999). Thus, it enhances the activity of NADH oxidase and NADH peroxidase. Administration of *P. freudenreichii* culture resulted in significantly increased percentage of bifidobacteria in fecal flora of humans as well as stool frequency (Kaneko 1999).

Propionic acid bacteria intake results in a significant increase in bifidobacteria counts and general improvement of intestinal microbiota (Hojo et al. 2002). There is evidence on synergistic effect of propionic acid bacteria species in co-cultures with other probiotics. In a study on human intestinal cell culture *P. jensenii* 702 was shown to promote adherence of bifidobacteria (Moussavi and Adams 2010).

2.1.4.3 Antimicrobial activity

Propionibacterium spp. strains express antimicrobial activity mainly against Gram-negative spp. (Yuksekdag et al. 2014), as well as antifungal activity (Ho et al. 2009). *P. freudenreichii* ssp. *shermanii* JS was shown to aggregate with pathogenic cells, preventing their colonization and infection of GIT (Collado et al. 2008).

Dairy propionic acid bacteria were found to inhibit *Helicobacter pylori*. *H. pylori* is a pathogen that causes chronic gastritis and is a risk factor for gastric cancer. Eradication therapy by combination of antibiotics brings about detrimental alterations in gut microbiota. However, it is possible to counteract these effects through probiotic treatment. *P. freudenreichii* ssp. *shermanii* JS was used in with other probiotics for patients undergoing *H. pylori* eradication therapy. Administration of probiotics improved tolerance to eradication treatment as compared to placebo group and increased recovery of probiotic bacteria in feces was achieved (Myllyluoma et al. 2005). This strain inhibited adhesion of *H. pylori* to epithelial cells and hindered *H. pylori*-induced release of interleukin 8 *in vitro*, which constituted anti-inflammatory effect. (Myllyluoma et al. 2008).

2.1.4.4. Immunomodulation, anticancerogenic and antimutagenic activities

Immunomodulatory activity of *P. freudenreichii* ssp. *shermanii* JS was demonstrated also in adults. In healthy adults receiving probiotic-containing drink for three weeks, serum level of C-reactive protein (a sensitive marker of inflammation) was decreased (Kekkonen et al. 2008).

Propionic acid bacteria were shown *in vitro* to reduce effects of a number of cancer risk factors including mycotoxins, cyanotoxins, plant lectins, some heavy metals such as cadmium and lead. Since they are able to reduce gut absorption of these compounds *in vivo*, the risk of colorectal cancer may be lowered (Cousin et al. 2011).

Conjugated linoleic acid (CLA), an isomer of linoleic acid with conjugated double bond, which possesses biological activity, is mainly derived from dairy products (Bhattacharya et al. 2006). In numerous *in vitro* and animal models CLA have been shown to have several health benefits: anticarcinogenic, antiatherogenic, antiadipogenic, antidiabetogenic and anti-inflammatory properties. Thus, CLA-enriched products are of high interest (Bhattacharya et al. 2006). CLA production by microorganisms is strain dependent and many strains of propionic acid bacteria are CLA positive, including strains of both *P. freudenreichii* ssp. *shermanii* and *freudenreichii* (Andrade et al. 2012).

Short-chain fatty acids (SCFA) produced by propionic acid bacteria (namely propionate and acetate) were shown to induce apoptosis of colorectal carcinoma cells. *In vitro* studies showed that acetate and propionate directly affect mitochondria resulting in loss of transmembrane potential, mitochondrial swelling and generation of reactive oxygen species (Jan et al. 2002).

Dairy propionic acid bacteria also demonstrated antimutagenic activity, i.e. reduced levels of spontaneous and induced mutations (Vorobjeva and Abilev 2002). Vorobjeva et al. demonstrated that propionic acid bacteria suppress mutations caused by 4-nitro-quinoline and N-nitro-N-nitrosoguanidine as well as 9-aminoacridine and 2-nitrofluorene (Vorobjeva et al. 2008). The mechanisms involve protein factor but are not fully elucidated yet (Vorobjeva and Abilev 2002).

2.1.4.5 Propionic acid bacteria as probiotics in fermented foods

In the light of probiotic potential propionic acid bacteria species were employed for food fermentations other than cheese. Propionic acid bacteria were proposed as delivery vectors facilitating adaptation of propionic acid bacteria to digestive stresses and enhancing the efficacy of action (Cousin et al. 2012).

An early work by LeBlanc et al. (2006) used *P. freudenreichii* NIZO B2336, a spontaneous roseoflavin-resistant mutant derived from *P. freudenreichii* B374, in combination with commercial starter culture to produce yogurt. The mutant strain is characterized by high

riboflavin (vitamin B2) production. Propionic acid bacteria counts in yogurt were as high as 5×10^8 cfu/g. The product fed to rats resulted in improved riboflavin status of previously depleted animals (LeBlanc et al. 2006).

In recent work Farhadi et al (2013) inoculated milk with adjunct starter cultures with *P. freudenreichii* ssp. *shermanii* and *L. acidophilus*. The study was focused on production of propionic acid which can be used as natural food preservative. *P. freudenreichii* maintained count of 9×10^6 cfu/ml which proved fermented milk environment to be good environment for bacteria. Fermentation temperature 30°C and inoculation ratio 1:4 of *L. acidophilus* to *P. freudenreichii* ensured maximal count of the latter as well as highest level of propionic acid (Farhadi et al. 2013).

The first dairy product (fermented milk) exclusively fermented by *P. freudenreichii* was proposed by Cousin et al (2012). Since propionic acid bacteria grow poorly in milk, the milk needs to be supplemented with lactate and casein hydrolysate. The *P. freudenreichii* strain was cultured in the modified milk medium and the fermentation was performed for three days at 30°C. This resulted in population levels of 10^9 cfu/g, which is equal to counts reported for cheeses (Thierry et al. 2011). Propionic acid bacteria showed good tolerance to *in vitro* and *in vivo* stresses; the bacteria remained viable during cold storage at 4°C for at least 15 days, which is important for microorganism to be considered as probiotic. The effect of fermented milk *in vivo* was evaluated on piglets, demonstrating high survival rate of the tested *P. freudentreichii* strain in the gut evidenced by the presence of propionate and acetate formed by this strains (Cousin et al. 2012).

2.2 Vitamin B12: structure, biosynthesis and role

2.2.1 Vitamin B12 structure

Vitamin B12 was first found to be a curing factor for pernicious anemia in 1926. It was isolated and crystallized in 1948. And its structure was deeply characterized by Dorothy Hodgkin in 1956 (Raux et al. 2000, Warren et al. 2002). Vitamin B12 (cobalamin) belongs to the group of compounds called corrinoids. Corrinoid molecule consists of two basic parts: planar corrin ring in the center and nucleotide at a right angle to it (Fig. 2). Corrin ring is comprised of four pyrroles. Two of them (rings A and D) are linked directly to each other which is a unique feature of corrinoids that distinguishes them from porphyrins, e.g. heme. Corrin ring is coordinated around cobalt atom via bonds between pyrrol nitrogens

and metal. Nucleotide is a lower axial ligand of cobalt. Cobalamin is the form of corrinoids in which nucleotide is represented by 5,6-dimethylbenzimidazole (DMBI). DMBI is covalently attached to cobalt via one of its nitrogens. The second nitrogen is linked to ribose phosphate. The latter is, in its turn, is attached to D-pyrrole ring through aminopropyl residue. Thus, a so-called nucleotide loop is formed (Warren 2006; Miller and Green 2007).

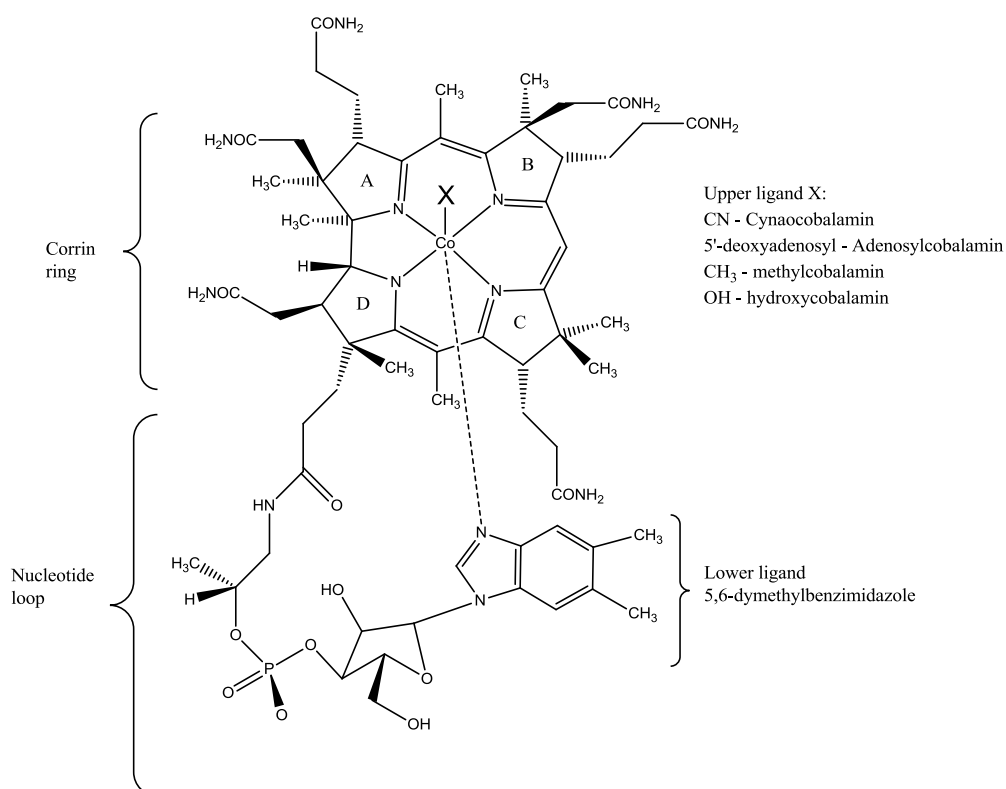


Figure 2. Cobalamin structure (Warren 2006; Miller and Green 2007)

Cobalamins as a group of compounds are distinguished by the identity of the upper ligand, the third part of the molecule. In coenzyme forms the upper ligand is mainly represented by 5'-deoxyadenosyl or methyl in adenosylcobalamin and methylcobalamin, respectively. Hydroxycobalamin, more rarely, but still can be a coenzyme form. Strictly speaking, the term vitamin B12 is restricted to cyanocobalamin which has CN group as an upper ligand. It is the most stable pharmacological form of the vitamin. All other forms are converted to cyanocobalamin in the presence of light and cyanide ions (Miller and Green 2007). In various Bacteria and Archaea several other forms of cobalamin are found, e.g. glutathionylcobalamin, sulfitocobalamin, and nitritocobalamin, but their functions are not yet well understood (Miller and Green 2007).

Apart from cobalamin, bacteria can produce a variety of cobamides with different lower ligands, including different benzimidazoles, purines and phenolic compounds (Fig. 3) (Hazra et al. 2013). The forms lacking nucleotide moiety at all are called cobinamides, e.g. cobinamide phosphate, guanosine diphosphate-cobinamide (GDP-cobinamide), which are formed under conditions unfavorable for benzimidazole synthesis. The form in which benzimidazole is replaced by adenine is called pseudo-B12. *P. freudenreichii* synthesizes incomplete forms as well as pseudo-B12 in small amounts (Vorobjeva 1999).

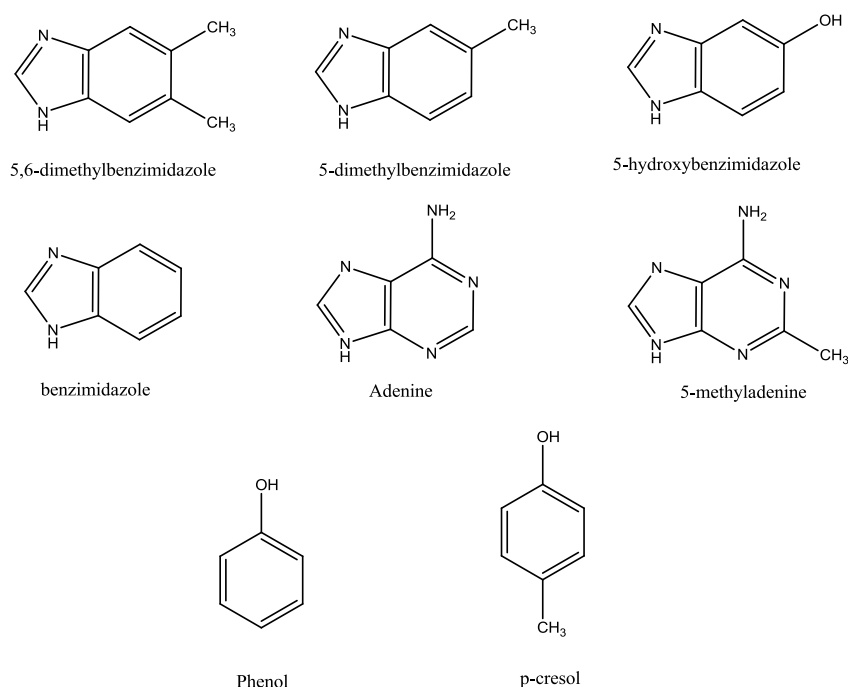


Figure 3. Lower ligands structures

The identity of lower base ligand affects binding catalytic ability of cobamide coenzyme forms (Hazra et al. 2013). Binding of cobamides occurs in two forms. In base-off form lower ligand is not coordinated to cobalt. Such cobalamin-dependent enzymes as methionine synthase, methyl-malonyl-CoA mutase bind coenzyme in this mode. Having no bond with cobalt, DMBI is replaced by histidine residue of apoenzyme (Ludwig and Matthews 1997). In base-on mode DMBI remains coordinated to cobalt. In this form cobalamin is bound to such enzymes as ribonucleotide reductase, diol dehydratase, etc. (Hazra et al. 2013).

2.2.2 Vitamin B12 biosynthesis

In nature, cobalamin synthesis is restricted to certain microorganisms only. A few genera are known to be B12 producers: *Aerobacter*, *Agrobacterium*, *Alcaligenes*, *Azotobacter*,

Bacillus, *Clostridium*, *Corynebacterium*, *Flavobacterium*, *Micromonospora*, *Mycobacterium*, *Norcardia*, *Propionibacterium*, *Protaminobacter*, *Proteus*, *Pseudomonas*, *Rhizobium*, *Salmonella*, *Serratia*, *Streptomyces*, *Streptococcus* and *Xanthomonas*. However, mainly *Pseudomonas denitrificans* and *Propionibacterium freudenreichii* are of industrial importance. The advantage of *P. freudenreichii* is the fact that it has GRAS status, therefore cobalamin from them is food grade (Martens et al. 2002).

The components of complex B12 molecule (corrin ring, upper ligand and nucleotide) are synthesized separately followed by assembly. Biosynthetic pathway of cobalamin at early steps is common to other tetrapyrroles which have corrin ring in the structure, including haem, chlorophyll, sirohaem and coenzyme F430. The common precursor of the core structure is 5-aminolaevulinic acid (ALA) (Fig. 4). It is converted to uroporphyrinogen III (UroIII) and at this stage B12 synthesis follows pathway which is separate from haem and chlorophyll (Roth et al. 1996; Raux et al. 2000).

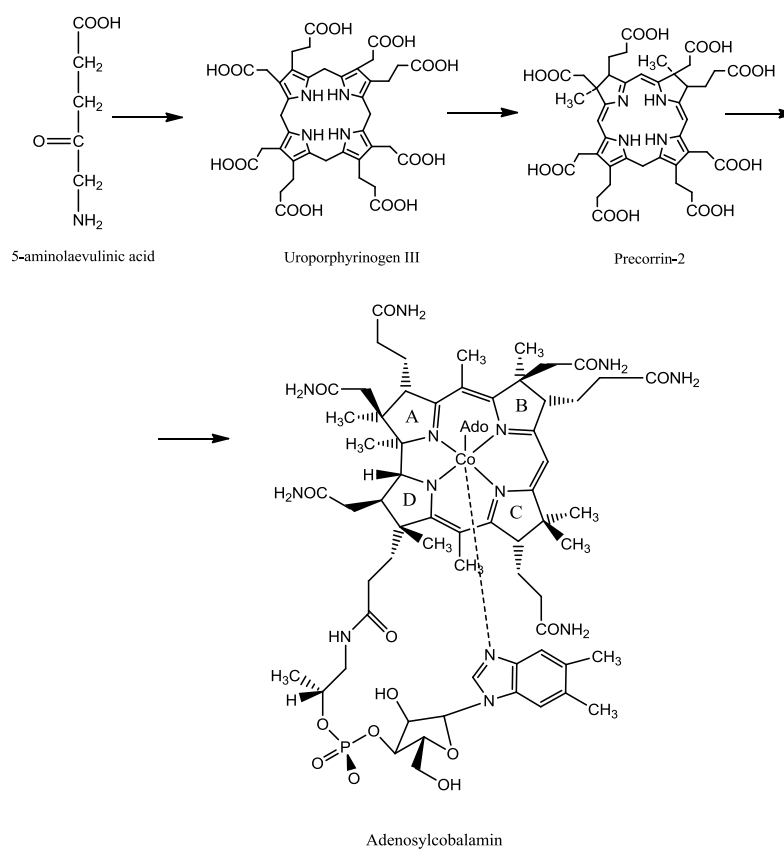


Figure 4. Cobalamin biosynthesis overview (Raux et al. 2000; Warren et al. 2002)

B12 biosynthesis pathway can be divided into three steps: synthesis of corrin ring, synthesis of nucleotide, assembly of the components (Raux et al. 2000). The whole synthesis includes around 30 enzyme-catalyzed steps (Vorobjeva 1999; Raux et al. 2000). Corrin ring is formed resulting from transformation of uroporphyrinogen III. This transformation includes peripheral methylations and ring contraction, which results in direct linking of pyrroles A and D (Raux et al. 2000; Warren 2006).

The stage of corrin ring formation can be accomplished by one of the two distinct pathways by bacteria: anaerobic and aerobic (Fig. 5) (Raux et al. 2000; Warren et al. 2002). This separates bacteria into two types. *P. freudenreichii* as well as *Salmonella typhimurium* and *Bacillus megaterium* synthesize B12 using anaerobic pathway. *Pseudomonas denitrificans* is a well-studied aerobic B12-producer (Martens et al. 2002). The difference between these pathways is in the timing of cobalt insertion and the mechanism of ring contraction (Warren et al. 2002). Accordingly, the pathways contain specific enzymes (Rodionov et al. 2003).

In anaerobic pathway used by *P. freudenreichii* cobalt insertion occurs at an early stage when the ion is inserted to the intermediate called precorrin-2 (Fig. 5). Cobalt is thought to mediate catalysis of subsequent ring contraction. By contrast, in aerobic pathway cobalt insertion takes place at the late stage after all major transformations in precorrin ring are completed (Raux et al. 2000; Warren et al. 2002).

Anaerobic and aerobic pathways proceed the same way from the step of adenosyl-cobyric acid (in case of adenosylcobalamin formation). It is converted to cobinamide by attachment of aminopropanol moiety. Then, cobinamide is activated via phosphorylation and formation of GDP-cobinamide. The last step of coenzyme formation is the attachment of the lower ligand base (DMBI). Before that, DMBI is activated by the attachment of phosphoribosyl moiety. The resulting compound is joined to GDP-cobinamide forming the nucleotide loop and releasing GMP (Warren et al. 2002; Martens et al. 2002).

The activation of DMBI base is regulated by *cobT* gene encoding nicotinate mononucleotide (NaMN):base phosphoribosyltransferase. It catalyzes formation of alpha-ribazole-5'-phosphate from nicotinate mononucleotide and DMBI. In *P. freudenreichii* CIRM-BIA1^T genome *cobT* is fused with *bluB* gene which encodes nitroreductase (Falentin et al. 2010). Database search in UniProtKB showed that this fusion gene

BluB/CobT has been reported only in *P. freudenreichii* CIRM-BIA1^T and *Kitasatospora setae* so far.

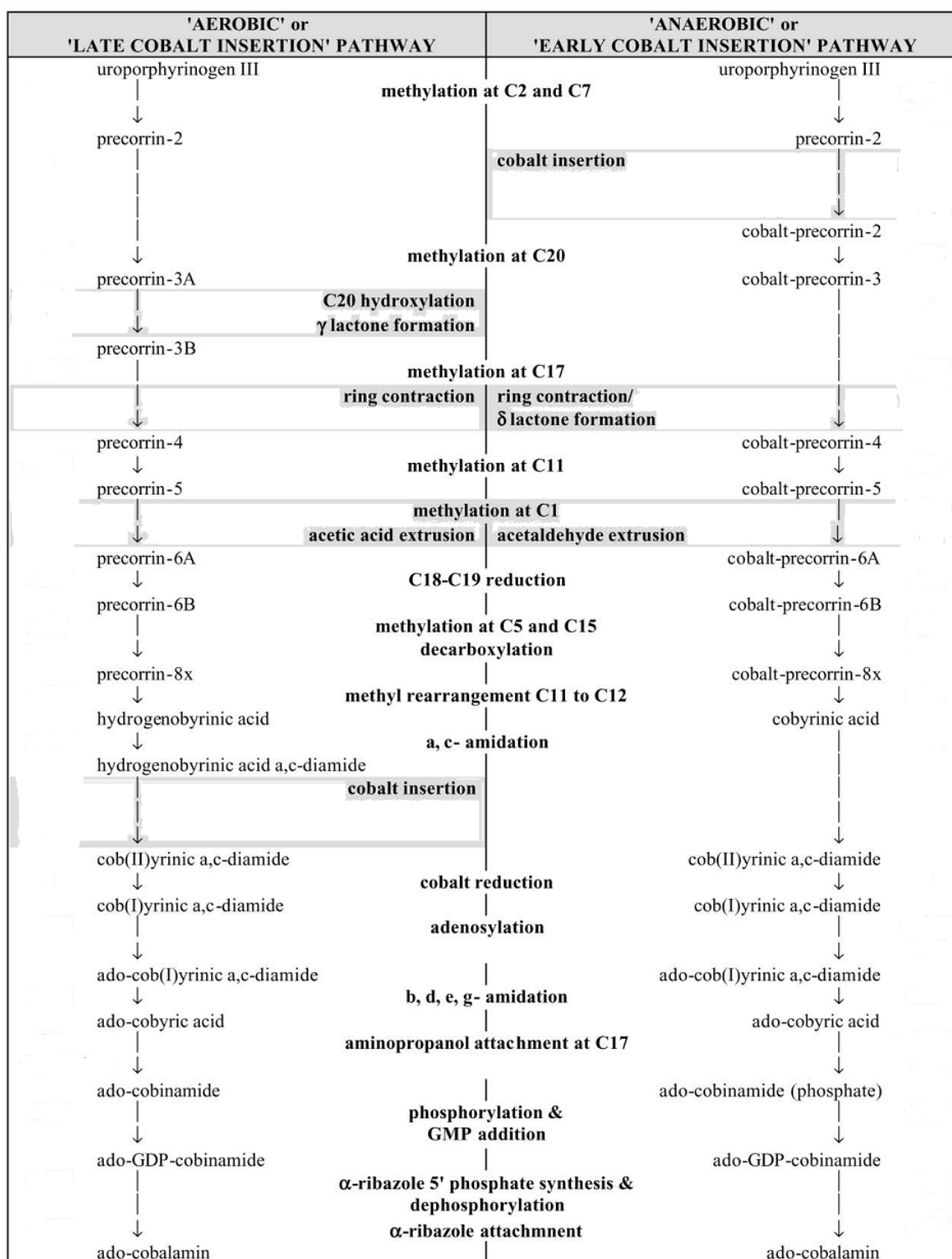


Figure 5. Aerobic and anaerobic cobalamin biosynthetic pathways (adapted from Warren et al. 2002)

BluB gene product emerged as a DMBI synthesizing enzyme. However, the mechanism of the synthesis has not been elucidated yet (Taga et al. 2007). Recent studies showed that CobT enzyme might be responsible for structural diversity of cobamides. The enzyme was shown to phosphoribosylate benzimidazole, purine and phenolic bases (Cheong et al. 2001; Hazra et al. 2013). *CobT* gene product appears to lack substrate specificity which results in the diversity of cobamides that can be synthesized by one species (Chan et al. 2014). However, in a recent study seven CobT homologues were tested with a variety of bases and six of them exhibited preference for DMBI if it was present in the environment (Hazra et al. 2013; Crofts et al. 2013).

2.2.3 DMBI biosynthesis: anaerobic and aerobic pathways

DMBI is an important component in the industrial production of cobalamin as it promotes formation of vitamin B12. It is used to for supplementation of medium and can be added at different stages of fermentation. Bacteria utilize exogenous DMBI complete cobalamin biosynthesis by attaching it to cobinamide (Wang et al. 2012). Biosynthesis of DMBI by bacteria themselves remains elusive although some recent findings enable better understanding.

Two biosynthetic pathways of DMBI are known: anaerobic and aerobic. Bacteria utilizing anaerobic pathway use glycine, glutamine, formate, erythrose-4-phosphate, and methionine for DMBI synthesis. This pathway was studied in strict anaerobe *Eubacterium limosum*. Glycine, formate and erythrose-4-phosphate donate carbons, whereas nitrogen is derived from glutamine (Renz 1998).

In species synthesizing DMBI only aerobically it is produced via enzyme-catalyzed pathway from riboflavin. It was first suggested that DMBI is derived from riboflavin as they have common structure. Radiolabelling studies showed that radioactivity from riboflavin was incorporated into vitamin B12 and, more specifically, to DMBI moiety (Renz 1970). Later it was specified that it is 1'-¹⁴C in riboflavin molecule that is transferred to C-2 position of DMBI as it was shown in *P. freundenreichii* (Renz and Weyhenmeyer 1972). This conversion was thought to be catalyzed by a constitutive enzyme system although no enzyme involved was recognized (Renz 1998). Horig and Renz showed that flavin mononucleotide (FMN) is an immediate substrate for DMBI formation (Horig and Renz 1980).

2.2.4 BluB – DMBI-synthesizing enzyme

The enzyme for oxygen-dependent conversion of riboflavin to DMBI through FMN was unknown until 2006, when Campbell et al. suggested that BluB enzyme catalyzes this reaction. BluB was first proposed to be involved in the conversion of cobinamide to cobalamin in *Rhodobacter capsulatus* (Pollich and Klug 1995). The enzyme acquired its name as *blu* genes play role in ‘blushing’ of *R. capsulatus* after reduction of partial O₂ pressure (Warren 2006). The *bluB* gene was previously suggested to encode a candidate cobalt reductase and was identified in various cobalamin-producing species (Rodionov et al. 2003).

In the research by Campbell et al. the production of acidic polysaccharide succinoglucan by nitrogen-fixing bacterium *Sinorhizobium meliloti* was investigated. It was found that the mutants exhibiting unusually bright fluorescence on calcofluor-containing medium had an inactivated gene that showed 36% homology to *bluB* gene of *R. capsulatus*. The mutants appeared to be unable to grow either in the absence of DMBI or only in presence of cobinamide. They were found to synthesize an intermediate (GDP-cobinamide) unless DMBI was provided. *BluB* mutants of *S. meliloti* were the first DMBI auxotrophs reported. Thus, it was concluded that BluB is required for the aerobic synthesis of DMBI (Taga et al. 2007).

BluB is an enzyme which belongs to NADH/FMN-dependent nitroreductase family (Campbell et al. 2006). The enzyme was crystalized and structure investigated by the same research group. It exhibited structural similarity to flavin oxidoreductase and functional relatedness to mono-oxidases (Taga et al. 2007).

Taga et al. also reported that it was a reduced form of FMN (FMNH₂) that was consumed by BluB, although a separate enzyme is responsible for the NAD(P)H-dependent reduction of FMN. BluB itself was found to cleave ribityl tail of FMN yielding D-erythrose 4-phosphate (Fig. 6). The activity of BluB includes breakage of three bonds and formation of one as well as contraction of six-membered ring of FMNH₂ yielding a five-membered ring of DMBI. For this oxygen-dependent reaction no NAD(P)H appeared to be required. Furthermore, the enzyme was assigned to a new family of ‘flavin destructases’ (Taga et al. 2007).

BluB protein was soon also identified in *Rhodospirillum rubrum*. *BluB* mutants of this bacterium appeared to be deficient in the reaction of bacteriochlorophyll biosynthesis catalyzed by B12-dependent enzyme. The activity was restored by addition of DMBI, cobalamin or introduction of plasmid encoding the wild type allele of *bluB*. Thus, BluB was established to be necessary and sufficient for oxygen-dependent conversion of FMNH₂ to DMB. No metals or organic factors were required. In contrast to *S. meliloti*, *R. rubrum* due to lack of BluB function accumulated cobyric acid, an intermediate of de novo corrin biosynthetic branch of the pathway (Gray and Escalante-Semerena 2007).

In the research by Wang and Quan (2011) the identification of BluB-catalyzed reaction mechanism was explored by hybrid density functional method. The analysis allowed distinction of two separate stages of the reaction and divide it into at least 14 steps. The first stage is thought to involve incorporation of oxygen followed by fragmentation of alloxazine ring of FMNH₂ yielding alloxane and ribityl dimethylphenylenediimine (Fig. 6). The second stage can include at least ten steps which leads in the formation of DMBI and release of D-erythrose 4-phosphate. Thus, relatively simple structure of BluB enzyme appears to contradict the complexity of the described multifunctional catalysis (Wang and Quan 2011).

Yu et al. investigated the diversity of *S. meliloti* mutants which had point mutations located in conserved residues in the active site of BluB. The domain has a unique structure of 'lid' that is thought to shield the active site from solvent. Some mutants demonstrated weakened binding affinity to both FMN and FMNH₂. The mutants also had severely reduced DMBI synthesis, thus, exhibiting loss of BluB catalytic function. This showed the importance of active site residues for DMBI biosynthesis (Yu et al. 2012).

In recent work Collins et al. first demonstrated *bluB* expression in species utilizing anaerobic pathway of B12 biosynthesis, namely *Bacillus megaterium*. Species discussed before (*S. meliloti*, *R. rubrum*) synthesize B12 under aerobic conditions. This work also first experimentally demonstrated an intermediate of FMN destruction, 4a-peroxyflavin. However, it was only found in reaction by *R. capsulatus* (Collins et al. 2013).

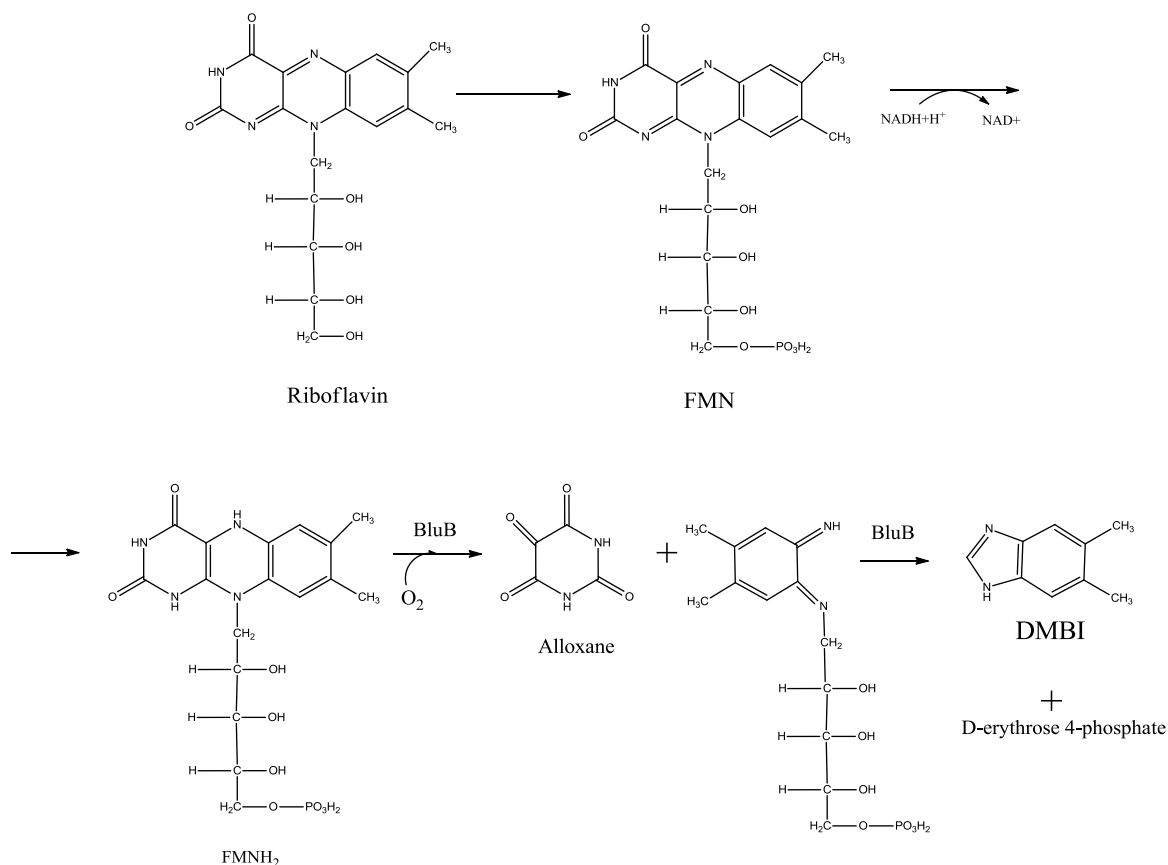


Figure 6. BluB-catalyzed oxygen-dependent DMBI synthesis

Experiments by Maggio-Hall et al. (2003) presented facile non-enzymatic conversion of riboflavin to DMBI in a cascade of oxidative reactions. This phenomenon was thought to be responsible for low DMBI levels found in *S. meliloti bluB* mutant strains (Yu et al. 2012).

2.2.5 Improvement of vitamin B12 production by *P. freudenreichii*

Cobalamin productivity is strain-dependent. Thirty seven strains of *P. freudenreichii* were screened for B12 production by Hugenschmidt et al. (2010). Cobalamin levels were in the range 0-2.5 µg/ml (Hugenschmidt et al. 2010). Berry and Bullerman (1966) reported the level as high as 15 µg/ml in a two-step process (Berry and Bullerman 1966).

The improvement of B12 production by *P. freudenreichii* can be achieved by three ways:

- 1) Two-step production with aeration provided at the late stage of incubation
- 2) Addition of DMBI
- 3) Genetic manipulation

Industrial cobalamin production using *P. freudenreichii* is conducted in a two-step process. First, in anaerobic stage, cobinamide formation takes place. Then, the culture is transferred to aerobic condition when DMBI moiety is synthesized and attached to cobinamide yielding coenzyme form (Speedie and Hull 1960; Martens et al. 2002). This switch is also governed by growth regulation needs. Oxygen is known to be an essential factor in growth of B12-producing strains of *P. freudenreichii*. Large amounts of propionate accumulated under anaerobic condition naturally inhibit the bacteria. When aerated, propionate in the media decomposes eliminating inhibition factor. Higher cell density is achieved and volumetric vitamin productivity increases (Ye et al. 1996). Aeration appears to have a positive effect on its own and the level of air supply was not found to have effect on B12 productivity (Berry and Bullerman 1966).

In order to promote cobalamin synthesis in industrial fermentation, exogenous DMBI is introduced (Wang et al. 2012). Its optimal level was established to be 15 ppm. (Marwaha et al. 1983) although in some research the level as high as 10 ppm (Berry and Bullerman 1966) and 20 ppm (Yongsmith et al. 1982) were reported as ensuring highest B12 yields. DMBI was recommended to be added to culture medium at the late stage of growth.

Marwaha et al. reported that optimal infusion time of sterile DMBI is 24 h before the end of fermentation (with 168h as a total duration of the process). Earlier introduction of the precursor was found to suppress the growth of *P. shermanii* culture and higher levels of B12 could be achieved when DMBI infusion was delayed (Marwaha et al. 1983). However, the study by Wang et al. (2012) did not show inhibition of growth although it confirmed the importance of DMBI infusion timing for coenzyme B12 biosynthesis. The highest productivity was observed when DMBI was introduced to *P. freudenreichii* culture at 84 h of incubation. At this point the highest level of cobinamide was observed (Wang et al. 2012).

Manipulation of genes involved in cobalamin biosynthesis is another way of increasing vitamin production. Modified strains are able to produce levels as high as 300 mg/l. B12 productivity is commonly improved by random mutagenesis (Martens et al. 2002). Recombinant strains of *P. freudenreichii* expressing genes as *hemA* (encoding ALA-synthesizing enzyme), *cobA* (encoding uroporphyrinogen III methyltransferase), *cbiL* and *cbiF* (encoding precorrin-5 synthase and precorrin-8 synthase, respectively) were shown to

produce 1,5-2,2 fold higher levels of B12 depending on gene compared to non-recombinant one (Piao et al. 2004).

2.2.6 Vitamin B12 dependent enzymes

Cobalamin is known to be a cofactor for three types of enzymes: isomerases, methyltransferases and dehalogenases (Banerjee and Ragsdale 2003). In bacteria and archaea a number of important reactions are catalyzed by vitamin B12 dependent enzymes: acetogenesis, methanogenesis, ribonucleotide reduction, fermentation and dehalogenation (Banerjee and Ragsdale 2003).

In propionic acid bacteria, methylmalonyl-CoA mutase is of particular importance for Wood-Werkman cycle. The enzyme converts succinyl-CoA to methylmalonyl-CoA (Thierry et al. 2011). Besides propionic acid fermentation, in *P. freudenreichii* vitamin B12 is known to be involved in DNA synthesis, ribonucleotide reductase and glutamate mutase activity, stabilization of thiol groups in enzymes, methylation of cytosine residues in DNA, etc. (Vorobjeva 1999).

In mammals, including humans, B12 is a cofactor of only two enzymes: methylmalonyl-CoA mutase and methionine synthase (Martens et al. 2002). Methylmalonyl-CoA mutase is an adenosylcobalamin dependent enzyme and participates in propionyl-CoA metabolism (Fig. 1). Propionyl-CoA is first carboxylated to form methylmalonyl-CoA. Then, the enzyme catalyzes conversion to succinyl-CoA which is then involved in tricarboxylic acid cycle (Fig. 7). Propionyl-CoA accumulation results from degradation of valine, thymine, methionine and odd chain fatty acids, etc. B12 deficiency brings about acidosis due to accumulation of methylmalonic acid (Martens et al. 2002).

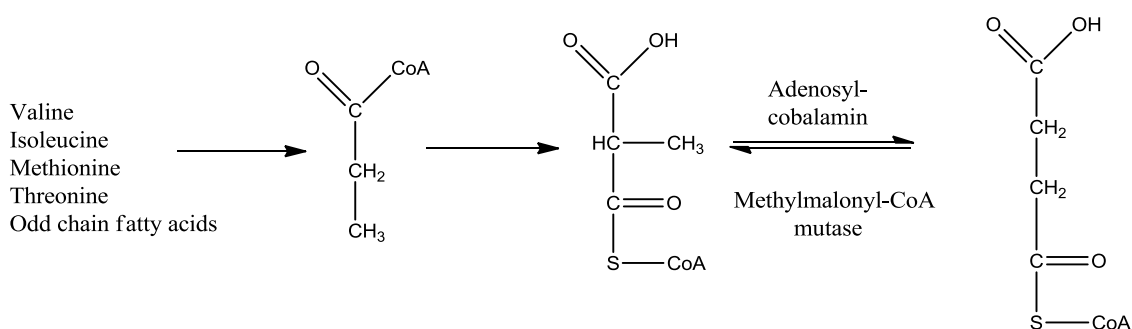


Figure 7. Methylmalonyl-CoA catalyzed reaction

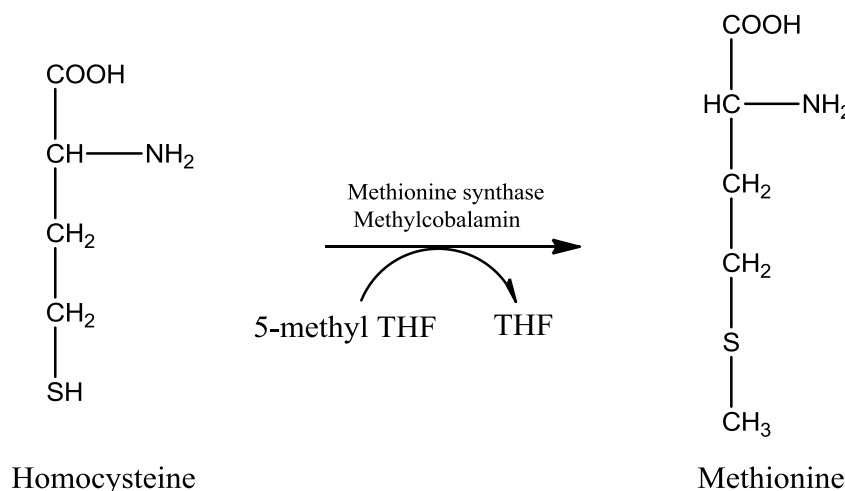


Figure 8. Methionine synthase catalyzed reaction

Methionine synthase is methylcobalamin dependent enzyme. It catalyzed transfer of methyl group from 5-methyltetrahydrofolate to homocysteine to form methionine (Fig. 8) (Ludwig and Matthews 1997). Vitamin B12 deficiency results in accumulation of homocysteine (Martens et al. 2002).

2.2.7 Vitamin B12 deficiency in humans

Cobalamin is essential for animals including humans. Due to inability to synthesize B12 we derive it from food sources. Plants and fungi are not known to use B12 in their metabolism or to produce it (Roth et al. 1996). Recommended intake for adults is 2.4 µg/day (FAO (WHO) 2002). Humans derive B12 mainly from food of animal origin: meat, milk, eggs, fish, and shellfish. Besides, some plant sources and mushrooms can contain substantial levels of B12 (Watanabe et al. 2013).

B12 deficiency is prevalent among elderly people. Besides, vegetarians, especially strict ones (vegans) are affected due to limitation or absence of animal derived foods in their diet. The problem also takes place in developing countries with high levels of poverty which does not allow consuming enough sources of vitamin B12 (Watanabe et al. 2013).

Indicators of B12 deficiency are decreased serum level of cobalamin as well as increased blood levels of methylmalonic acid due to impaired methylmalonyl-CoA mutase activity and homocysteine (also in urine) due to inactivation of methionine synthase (Miller and

Green 2007). Cobalamin deficiency mainly affects blood (results in anemia) and nervous system (causing neuronal demyelination) (Miller and Green 2007).

Deficiency in elderly people is often connected with malnutrition, malabsorption due to gastric and intestinal causes. Chronic gastritis, gastric atrophy and other problems cause impaired B12 absorption. All the disorders associated with intestine, especially terminal ileum (site for physiological absorption of cobalamin), also cause B12 deficiency (Miller and Green 2007).

Prevention of deficiency in vegetarians and elderly can be achieved through diet. Vegetarians can include plant-derived food enriched with B12: soyabean-fermented foods, edible algae (laver), various edible mushrooms (black trumpet and golden chanterelle), some vegetables (broccoli, asparagus, Japanese butterbur, mung bean sprouts, tossa jute, and water shield). For elderly people with B12 malabsorption it is recommended to consume B12-fortified products, e.g. cereals and milk (Watanabe et al. 2013).

2.3 2D electrophoresis as a tool for investigation of bacterial proteome

2.3.1 Principle of 2D electrophoresis

The term ‘proteomics’ was introduced in 1996 and refers to the study of overall protein pool produced in a cell, tissue or an organism (Rogowska-Wrzesinska et al. 2013). There are two main approaches on bacterial proteome investigation: two-dimensional gel electrophoresis (2D GE) and gel-free shotgun proteomics (Chao and Hansmeier 2012).

2D GE involves separation of proteins in two stages. First dimension is separation of proteins according to their net charge, or isoelectric point, in the process named isoelectric focusing (IEF). The second stage is sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), which is separation according to molecular weight (M_w) (Chao and Hansmeier 2012). The workflow of proteome investigation using 2D-GE is as follows (Rabilloud and Lelong 2011):

- 1) Sample preparation
- 2) IEF
- 3) SDS-PAGE
- 4) Visualization
- 5) Image analysis, detection of spots with differential abundance
- 6) Proteins identification

Sample preparation is a crucial step in gel-based protein separation which involves solubilization and purification of proteins. This is achieved by using a combination of chaotropic compounds and neutral detergents. Since proteins charge must not be changed uncharged chaotropes such as urea coupled with thiourea are used. Combination of urea and thiourea disrupts hydrogen bonds resulting in protein unfolding and prevention of interactions between molecules (Lopez 2007). To improve dissolving of cellular lipids and maintaining proteins soluble during IEF, electrically neutral detergent 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) is added. CHAPS is a surfactant that prevents protein molecules from hydrophobic interactions, aggregation and precipitation (Posch 2008; Rabilloud and Lelong 2011). Hydrophobic proteins, such as membrane-associated ones, are the hardest to solubilize. Hence, they can be excluded from the analysis. The use of SDS, which is an excellent solubilizing agent, would assist in dissolving membrane-associated proteins. However, SDS is incompatible with IEF (Lopez 2007).

At present, IEF is conducted using immobilized pH gradient strips (IPG) which improves reproducibility. There are several pH ranges available commercially from very narrow (pH 4-5) to very broad (3-10) (Lopez 2007). Prior to transfer to SDS-PAGE, IEF gels are equilibrated in SDS-containing buffer. SDS binds to the protein molecules and renders them the same net charge density. Proteins become mobile in polyacrylamide gel in the second dimension. Equilibration buffer contains dithiothreitol (DDT) which reduces disulfide bridges, thus unfolding tertiary structure. It is also necessary to protect sulfhydryl groups from oxidation. For this, iodacetamide is added to equilibration buffer (Drabik et al. 2013).

Visualization of separated proteins in the gels can be achieved by using various staining techniques. The most applied are Coomassie Brilliant Blue, silver and fluorescent dyes (Lopez 2007). Silver staining is very sensitive with the detection limit as low as 0.1 ng protein. However, it has narrow dynamic range and it is the least reproducible of all stains (Drabik et al. 2013). Nevertheless, silver staining is useful for qualitative determination of spots (presence/absence) (Lopez 2007). Coomassie Brilliant Blue is an organic dye which shows moderate sensitivity (8-10 ng) (Drabik et al. 2013) as well as good linearity and excellent compatibility with mass spectrometry (Rabilloud and Lelong 2011) which is now widely used for proteins identification. Fluorescent dyes, e.g. Sypro Orange used in this work, appear to be a compromise variant of the first two. Its sensitivity is as low as 1 ng

(Drabik et al. 2013). And it shows excellent linearity and is compatible with mass spectroscopy (Rabilloud and Lelong 2011).

2.3.2 Capacities and limitations of 2D GE

Proteomics nowadays is an indispensable method for large-scale analysis of physiology and metabolism in microorganisms. It enables understanding of physiological responses to a variety of stimuli. Proteomic approach was applied to study the mechanisms of physiological response to high and low temperatures, acid stress, organic solvents, oxidation, etc. (Han et al. 2011).

2D GE method allows to visualize up to a few thousands protein spots simultaneously (Drabik et al. 2013). 2D GE proteomics enables analysis of post-translational modifications: phosphorylation, glycolization as well as modification by oxidative stress (Sa-Correia and Teixeira 2010; Chao and Hansmeier 2012). Modifications, such as phosphorylation, glycolization, acetylation and deamidation, induce shift in pH value of proteins. This results in dislocation of spot on the proteome map (Rabilloud and Lelong 2011).

2D GE also enables quantitative analysis. It can be achieved via labelling as well as label free methods. Labelling methods include stable isotopes, isobaric mass tagging, chemical labelling (Han et al. 2010). The most common technique now is application of isotope-coded affinity tagging reagents (ICAT). Proteins are labelled with heavy and light ICAT reagents. After trypsin digestion quantification is accomplished based on relative signal intensities of identical peptides (Phillips and Bogoy 2005).

2D GE is a powerful tool for investigation of bacterial proteomes which are comparatively simple. However, the proteins that 2D-GE is able to resolve are those that are highly abundant and soluble. They perform limited number of basic functions, i.e. central metabolism, protein synthesis, degradation, stress response (Rabilloud and Lelong 2011).

One of the important shortcomings is low ability to resolve hydrophobic proteins, especially membrane-associated ones (Rabilloud and Lelong 2011). These proteins are involved in some important functions, such as adhesion, signal transduction, ion transport. Hydrophobic proteins are estimated to account for about 30% of cell proteome (Drabik et al. 2013). Gel visualization techniques limit the ability to detect low abundance proteins that may be present in a few copies per cell. Basic proteins are often missed out in 2D gels.

However, they may constitute a significant part of bacterial proteome (Lopez 2007). Commercially available IPG strips for basic pH range are known to have lower resolution compared to acidic strips (Drabik et al. 2013).

2.3.3 Proteomic studies on *P. freudenreichii*

The published proteomic studies on *P. freudenreichii* were mainly focused on its adaptability to abiotic and biotic stresses. This is due to probiotic potential of the species. Probiotics intended for use in food should tolerate technological stress (temperature, osmotic stress, acidity) and then they should tolerate conditions of gastrointestinal tract (gastric acid and bile salts) (Gagnaire et al. 2009).

An early study by Jan et al. (2001) was focused on acid stress adaptation by *P. freudenreichii* ssp. *shermanii* SI41. The study demonstrated the ability to develop acid tolerance response (ATR) by the cultures exposed to sublethal pH of 5.0 for 60 min. The procedure induced ATR development by bacterial cells. This resulted in maintenance of viability and high survival rates during challenge at lethal pH 2.0 as opposed to non-adapted cells. During adaptation treatment the total of 433 proteins separated by 2D GE were found to be synthesized. The synthesis had a time-dependent pattern. Proteins could be divided into those induced at early or late growth stage, as well as transiently or permanently. The protein pool induced by acid stress included general stress response proteins GroEL, GroES, which were synthesized at the late stage of adaptation. Another group of proteins relating to early response included enzymes involved in DNA synthesis and repair (RecR and RepB) and biotin carboxyl carrier protein (BCCP). BCCP is involved in decarboxylation of methylmalonyl-coenzyme A and carboxylation of pyruvate, the key reactions of Wood-Werkman cycle (Jan et al. 2001).

Leverrier et al. (2003) investigated bile salt stress on the same strain of *P. freudenreichii*. Adaptation strategy was also applied when cells were exposed to solution containing 0.2 g bile salts/l. This enabled to achieve high survival rate at lethal concentration of bile (1.0 g bile salts/l), the level exceeding that in a healthy human gut. Protein production was investigated after adaptation. Pre-treatment was found to induce 24 proteins of which five were synthesized de novo. Bile stress induced proteins included general stress response proteins such as DnaK, Hsp20, ClpB as well as BCCP and RecR, previously associated with acid stress. There were also proteins assigned to oxidative stress adaptation: superoxide dismutase SodA and cysteine synthase CysK. Another group of proteins was

proposed to be responsible for signal sensing and gene expression regulation: histidine kinase, alternative sigma factor AlgU and RpoE (Leverrier et al. 2003).

Follow-up study by Leverrier et al. (2004) covered three types of stresses: acid, bile salts and heat and common pathways of stress response were found for them. Six proteins were found to be induced commonly by different stresses, including aforementioned conserved heat shock proteins DnaK, ClpB, SodA and BCCP. Acid stress was specifically found to trigger the overproduction of enolase, malate dehydrogenase, GroEL, pyruvate flavodoxin-ferredoxin oxidoreductase and elongation factor EF-Tu involved in polypeptide metabolism. The following proteins were specifically induced by bile stress: as previously, superoxide dismutase and cysteine synthase, and ABC transporter OppD. Heat stress did not induce many specific proteins. Only a single-strand binding protein was found to be overproduced specifically in this case and its role is to respond to DNA-damaging agents (Leverrier et al. 2004).

Anastasiou et al. (2006) demonstrated by transcriptome-level analyses the thermotolerance in *P. freudenreichii* is strain-dependent feature. Proteomic study was conducted on several native strains isolated from traditional Graviera Kritis, a Gruyere-type cheese. The manufacture includes heat treatment at 52-53°C, which requires microflora to be thermotolerant. In accordance with heat stress response, all the strains were categorized into heat tolerant and heat sensitive groups. The two groups had distinct but overlapping heat stress response patterns. Tolerant strains were found to activate molecular chaperones, antioxidant protection and stringent response mechanisms. Overproduction of the following proteins was detected: chaperonin Cpn60, ClpB homologue, pyruvate-flavodoxin reductase, S-adenosylmethionine synthase (MetK), pyridine nucleotide-disulphide oxidoreductases, polyribonucleotide: ortho-phosphate nucleotidyltransferase. By contrast, sensitive strains tended to adapt through activation of signal translation, biosynthetic pathways, and cell wall maintenance and protein turnover. Heat stress induced overproduction of the following proteins: GroEL, DnaK, elongation factor EF-Tu GTP binding domain, enzymes synthesizing pyrimidine, thiamine, histidine, cysteine synthase, D-alanine-D-alanine ligase, H31 response regulator (Anastasiou et al. 2006).

A recent study of Dalmaso et al. (2012) addressed metabolism of *P. freudenreichii* in ripened cheese during cold storage at around 4°C, since it is known to remain active in this condition and continue to utilize nutrients and produce flavor compounds (Thierry et al.

2011). Responses at transcriptome-levels as well as at protein production levels during cold storage were studied. The investigation was conducted not in cheese but in a system mimicking ripening cheese, with lactate as the only carbon source and rich in soluble nitrogen. Accordingly, energy metabolism, amino acids and protein synthesis genes were down-regulated and cold stress markers were overexpressed. Among them stress response transcriptional protein PspC, CspA and CspB were found. Heat shock proteins were down-regulated: GroSL, DnaKJ, Hsp20, ElpB, GrpB. Volume of some proteins was found to be decreased: DnaK2, GroS1, ClpB2, Hsp20. Being affected by cold stress, metabolism was redirected towards glycogen synthesis through gluconeogenesis. Lactate dehydrogenase was maintained and pyruvate phosphate dikinase as well as glycogen synthesis enzymes were up-regulated. In addition, the maintenance of Wood-Werkman cycle, fat hydrolysis and ester synthesis were evidence of cells activity (Dalmasso et al. 2012).

Thus, proteomic studies on dairy propionic acid bacteria are in infancy with limited number of conditions investigated. Due to probiotic potential and various health benefits that these bacteria demonstrate, more investigation is required towards their physiological activities.

3. EXPERIMENTAL RESEARCH

3.1 AIM OF RESEARCH

The aim of this work was to investigate the effect of DMBI on vitamin B12 synthesis and protein production in *P. freudenreichii* spp. *freudenreichii* DSM 20271. For this the following objectives were achieved:

- 1) Vitamin B12 production was determined in the presence and absence of DMBI in growth medium at mid-logarithmic growth stage, and overall vitamin B12 productivity was evaluated after 168 h incubation. The effect of two-step process on final cobalamin level was evaluated in comparison to fully anaerobic process.
- 2) Proteome maps of *P. freudenreichii* ssp. *freudenreichii* cultures grown in the presence and absence of DMBI were obtained at mid-logarithmic growth stage.

3.2 MATERIALS AND METHODS

3.2.1 Bacteria and culture conditions

P. freudenreichii ssp. *freudenreichii* strain DSM 20271 obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany) was investigated. This is a type strain of *P. freudenreichii* ssp. *freudenreichii* that is unable to metabolize lactose.

The strain was cultured on propionic acid (PPA) agar with lactate as a carbon source. The composition of PPA was as follows: 5.0 g tryptone (Sigma-Aldrich, Co, USA), 10.0 g Bacto™ yeast extract (Becton, Dickinson and Co, France), 15.0 g Bacto™ agar (Becton, Dickinson and Co, France) and 0.84% (w/v) sodium DL-lactate added in the form of 60% (w/w) syrup (Sigma-Aldrich, Co, USA) in 1 l MiliQ water. pH was adjusted to 7.3 by the addition of 2M NaOH (Mallinckrodt Baker B.V., Holland) in solid medium. The media were autoclaved at 121°C for 15 min.

Liquid PPA media was prepared analogously, but without the addition of agar. Liquid PPA with increased level of carbohydrate source contained 3% (w/v) sodium DL-lactate. The value of pH in this medium is usually adjusted to 6.7 prior autoclaving. However, since lactate decreases pH of the medium after autoclaving, the required pH was achieved by over-adjusting pH up to 7.4 prior to autoclaving. In PPA with standard level of lactate, pH was adjusted to 6.7. After autoclaving at 121°C for 15 min the resulting pH values in PPA with 0.84 and 3% lactate were 6.61 and 6.59, respectively.

Liquid PPA was supplemented with cobalt chloride (Sigma-Aldrich, Germany) at a level of 5 ppm (Berry and Bullerman 1966). The samples to be containing 5,6-dimethylbenzimidazole (Merck, Germany) as a precursor (samples DMBI+) were supplemented with DMBI at a level of 15 ppm (Marwaha et al. 1983) at incubation time 0. The required amounts of cobalt chloride and DMBI solutions were filtered using 0.2 µm Whatman® Nylon filter media (Schleicher&Schnell, USA) and added directly to sterile media before inoculation. For each condition (DMBI+ and DMBI-) six replicate cultures were prepared.

The cultures were grown anaerobically at 30°C. Three out of six cultures for each condition were grown for 168 h fully anaerobically. The other three were grown in a two-step fermentation process, including 72 h anaerobic incubation followed by transfer to

aerobic condition where the cultures were incubated for the rest 96 h. Anaerobic conditions were obtained by culturing the strains in anaerobic jars with Aerocult®A reagent (Merck KGaA, Germany). Aerobic condition was provided by leaving tube caps slightly loose.

3.2.2 Growth kinetics

The growth in liquid PPA was monitored by measuring optical density (OD) at 600 nm (OD_{600}) using spectrophotometer (Novaspec® II, Amersham Pharmacia Biotech, Sweden) and 1.5 ml cuvettes (Brand GMBH, Germany). The pH of the culture samples and media was measured with 744 pH meter (Metrohm Ltd, Switzerland).

For inoculation of liquid cultures, bacteria were first cultivated on PPA agar at 30°C for 3 days. Cells were harvested and re-suspended in 1 ml PPA to obtain OD_{600} of 0.01, which was used to inoculate the culture media (45 mL).

Three conditions were investigated: bacterial cells cultured in PPA, PPA supplemented with 3% lactate and 5 ppm cobalt chloride, PPA supplemented with 3% lactate, 5 ppm cobalt chloride and 15 ppm DMBI. For each sample 45 ml of media in 50 ml Cellstar® tubes (Greiner Bio-One GmbH, Germany) were inoculated and for or each condition, two biological replicates were analyzed. The optical density was determined every 4 hours until the growth stopped, i.e. no obvious increase in cell densities was observed. In addition, pH was measured in each sample at every 24 hours starting from inoculation point.

3.2.3 Culturing for B12 analysis

For B12 analysis, bacterial cells were harvested at two time points of growth: first, at the same time as for 2D GE analysis at the mid-logarithmic stage and, second time, after 168 h. For the first harvest, bacteria were cultivated in 250 ml glass bottles using 230 ml of liquid PPA medium. For both DMBI(+) and DMBI(-) conditions, three biological replicates were used. At the time of 168 h harvesting (OD_{600} 4.1-4.9), each cultures were divided into five 50 ml Cellstar® tubes (Greiner Bio-One GmbH, Germany). These samples (50 mL) were centrifuged for 10 min at $3220 \times g$ in the centrifuge 5810 R (Eppendorf, Germany) at 4°C and the cells were re-suspended in 5 ml of ice cold PBS buffer. Cell pellets of the same sample were combined in one tube and the cells harvested by centrifugation for 10 min, $18514 \times g$ at 4°C. The samples were stored at -20°C before analysis.

The procedure of harvesting at 168 h was analogous to 24 h harvest. However, due to the fact that the late stationary cultures appeared to give very soft pellets after second centrifuging pellets were transferred to 1,5 ml Eppendorf tubes and centrifuged at $21500 \times g$ for 10 min in the centrifuge Himac CT 15RE (Hitachi Koki Co., Ltd, Japan). This allowed more complete separation of supernatant, although the pellets were still softer compared to the ones obtained after 24 h incubation. The samples were stored at -20°C before analysis.

3.2.4 UHPLC quantification of vitamin B12 level

Extraction and UHPLC analysis were conducted according to Chamlagain et al. Intracellular total cobalamins were extracted from the cells as cyanocobalamin (Chamlagain et al. 2015).

Frozen cell pellets were thawed at room temperature. Approximately 0.1 g of cells was weighed in plastic thermoduric tube. 10 ml of extraction buffer (pH 4.5) composed of 8,3 mmol/l sodium hydroxide (Merck, Germany) and 20.7 mmol/l acetic acid (Merck, Germany) was added to the samples followed by pipetting 100 μl of 1% sodium cyanide solution (Sigma-Aldrich, Germany) to each sample. After vortexing the samples were incubated for 30 min in a boiling water bath and cooled on ice. Next, the samples were centrifuged using Hermle Z323 (HERMLE Labortechnik GmbH, Germany) at 8000 rpm for 10 min and supernatants were collected in clean plastic tubes. Pellets were re-suspended in 5 ml extraction buffer (pH 6.2), centrifuged again and supernatants were collected. pH of the combined supernatants was adjusted to 6.2, which was followed by filtering through a qualitative filter paper (\varnothing 90 mm, VWR, France) to 25 ml volumetric flasks. The flasks were filled up with extraction buffer (pH 6.2). Before UHPLC analysis samples were filtered through 0.2 μm Acrodisc® filters (Pall Corporation, USA) into 12 \times 32 vials (Waters, USA). The rest of the samples were transferred to plastic bottles and stored at -20°C .

The UHPLC system consisted of Acquity UPLC® HSS T3 column (1.8 μm , 2.1×100 mm) and a guard column, Waters Acquity™ Ultra Performance LC, photodiode array detector, sample manager and binary solvent manager (Waters, USA). Chromatographic data was acquired using Empower 2 software. The analysis was performed at column temperature of 30°C . Mobile phase was composed of 0.025% trifluoroacetic acid (TFA) (Sigma-Aldrich, Germany) in MilliQ water (solvent A) and 0.025% TFA in acetonitrile

(Sigma-Aldrich, Germany) (solvent B). The flow rate was set at 0.32 ml/min. Injection volume was 10 µl. The mobile phase was a series of gradient steps including the following proportions of solvent A:solvent B : range 0-0.50 min 95:5; range 0.50-5.00 min linear gradient of solvent B from 5 to 40%; range 5.00-6.00 min 60:40; range 6.00-7.00 min linear gradient of solvent A from 60 to 95%; range 7.00-10.00 min 95:5. Each sample was injected twice. Cyanocobalamin in effluent was detected at 361 nm.

The standard curve was prepared with cyanocobalamin solutions. Stock solution with the concentration of 0.2 mg/ml was made by diluting cyanocobalamin (Supelco; Bellefonte, USA) in 25% aqueous ethanol solution. The stock solution was stored in the dark at 4°C. The actual concentration was measured right before UHPLC analysis by a spectrophotometer. For spectrophotometric determination stock solution was 10-fold diluted with 25% aqueous ethanol solution. The absorbance was measured at 361 nm in duplicate with 25% aqueous ethanol solution as a blank. Cyanocobalamin level was calculated using the equation:

$$c(\mu\text{g} \cdot \text{ml}^{-1}) = \frac{A_{361} \cdot MW_{\text{CN-Clb}} \cdot 10}{28.1},$$

where A_{361} – absorbance of cyanocobalamin stock solution at 361 nm; $MW_{\text{CN-Clb}}$ – cyanocobalamin molecular weight (1355.4 g/mol).

3.2.5 Culturing and harvesting for 2DE analysis

The bacteria were cultured in 50 ml Cellstar® tubes (Greiner Bio-One GmbH, Germany) using 45 ml of PPA medium inoculated. Harvesting cell samples was conducted at the mid-logarithmic stage of growth (24 h post inoculation) with cultures having OD_{600} of 0.56 - 0.91. From each tube two aliquots of 10 ml were transferred to 15 ml tubes (Greiner Bio-One GmbH, Germany). The rest of the culture was left for the second B12 analysis (see below). Logarithmic cultures were centrifuged (10 min, $3220 \times g$, 4°C) using the centrifuge 5810 R (Eppendorf, Germany), and the pelleted cells were washed using 10 ml of 50mM ice cold Tris-HCl (pH 8.0) (PlusOne, GE Healthcare, Sweden) and centrifuged again as described above. For more complete removal of supernatant pellets were transferred to 1.5 ml Eppendorf tubes and centrifuged at $21500 \times g$ and 4°C for 10 min. Samples were stored at -20°C before analysis.

3.2.6 Protein extraction, solubilization, purification and quantification

During extraction, solubilization and purification, unless stated differently, cell pellets were kept on ice in order to avoid degradation of the proteins. Before extraction 50 μ l of 0.2 M Tris (PlusOne, GE Healthcare, Sweden) was added to cell pellets. After this the cell samples were transferred to 1.5 ml microcentrifuge tubes with skirt (Greiner Bio-One GmbH, Germany) and cells were broken using Fast Prep-24 (M.P. Biomedicals, USA) in presence of sterile glass beads $\varnothing = 0.1$ mm (Sigma-Aldrich). The lysis included 3×30 s cycles at the speed of 6.5 m/s. The pellets were held on ice in between the cycles for 1 min.

Proteins released after lysis were dissolved in 300 μ l UTC-T buffer composed of 7 M urea (GE Healthcare), 2 M thiourea (Merck KGaA), 4% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) (GE Healthcare) and 30 mM Tris-HCl (pH 8.0) (PlusOne, GE Healthcare, Sweden). Samples were incubated at room temperature for 10 minutes followed by centrifugation ($21500 \times g$, 20°C, 30 min) using centrifuge Himac CT 15RE (Hitachi Koki Co., Ltd, Japan).

Protein samples were then purified using 2D Clean-Up Kit (GE Healthcare, Sweden). To the protein solutions (at a volume of 220 μ l), 550 μ l of the precipitant was added, the mixtures were incubated on ice for 15 min with frequent vortexing to ensure protein precipitations. Then, 550 μ l of the co-precipitant was added to each sample, and the samples were briefly vortexed prior to centrifugation ($21500 \times g$, 10 min, 4°C). After removal of supernatants, 50 μ l of the co-precipitant was added without disturbing the pellet to cover the surface, and the tubes were centrifuged for 5 min at 4°C. Supernatants were discarded and 50 μ l of MiliQ water was added and the samples were vortexed to break the protein pellets. Finally, ice-cold washing buffer (1 mL) together with 5 μ l wash additive were pipetted to each sample, and the samples were then incubated at -20°C for 30 min with repeated vortexing every 10 min before further analysis.

Protein concentration in the samples was determined using the 2D Quant Kit (GE Healthcare, Sweden). Prior to quantification, wash buffer from purification step was removed after centrifugation at 4°C for 10 min. The residues of the buffer were dried for 2-3 min in the open tubes. Next, UTC-T buffer was added to the precipitated protein and vortexed until protein pellet was dissolved. The samples typically required 80 μ l of buffer. Protein solutions were centrifuged at 20°C for 10 min and supernatants were transferred to new tubes.

Quantitative assay was performed in triplicates according to protocol provided by manufacturer. For each sample 2 µl of protein solution were pipetted to a 1.5 ml tube, mixed with 500 µl precipitant from the kit, vortexed briefly and incubated for 2-3 min at room temperature. Next, 500 µl co-precipitant were pipetted into each tube followed by vortexing and centrifugation at 20°C for 5 min. Supernatant was thoroughly removed. To each tube 100 µl copper solution and 400 µl MiliQ water were added and mixed by vortexing to dissolve protein. Prior to assay an appropriate volume of working color reagent was prepared by mixing 100 parts of color reagent A from the kit with 1 part color reagent B. To each tube with protein sample 1 ml ready color reagent mix was pipetted and mixed by inversion followed by incubation for 15 min at room temperature.

The absorbance of samples was read at 480 nm in 1.5 ml cuvettes (Brand GMBH, Germany) by spectrophotometer ORDIOR UV-1800 (Shimadzu, Japan) using MiliQ water as reference. Standard curve was generated using bovine serum albumin (BSA) solution provided with the kit.

3.2.7 Two-dimensional gel electrophoresis

3.2.7.1 Isoelectric focusing

The IEF was performed using Ettan IPGphor 3 system (GE Healthcare, Sweden) with 11 cm Immobiline DryStrip gel strips (IPG) of pH 4-7 (GE Healthcare, Sweden). Prior to IEF, IPG strips were rehydrated for at least 12 h in DeStreak rehydration solution (220 µl) containing 1% IPG buffer (GE Healthcare, Sweden) using the rehydration chamber.

Samples to be loaded on IPG strips were composed to ensure equal amounts of protein (50 µg), 1% IPG buffer (GE Healthcare, Sweden) and 50 mM DTT (SIGMA-ALDRICH). In addition, 1.2 µl bromphenol blue (PlusOne, Amersham Biosciences) was added to color the sample. Total volume of each sample was 60 µl and that was achieved by adding UTC-T buffer.

Rehydrated IPG strips were placed on the IEF tray and the analyzed samples were applied using anodic cup-loading technique. The IEF-tray with loaded strips was filled with DryStrip Cover fluid (GE Healthcare, Sweden) to prevent drying of the strips. The loaded IPG strips were focused at 20 °C 500 V for 500 Vh, linear ramping to 1000 V for 800 Vh, linear ramping to 6000 V for 8800 Vh, hold at 6000 V for 2900 Vh, and step down to 500 V.

After IEF the IPG strips were equilibrated in 2 mL equilibration buffer A consisting of 50 mM Tris-HCl pH 6.8 (PlusOne, GE Healthcare, Sweden), 6 M urea (GE Healthcare), 2 % SDS (PlusOne, GE Healthcare), 20% glycerol (Merck KGaA, Germany), with 2 % (w/v) DTT (SIGMA-ALDRICH) for 15 min, followed by 15 min in 2 mL equilibration buffer B having the same composition except for having 2.5 % (w/v) iodoacetamide (Bio-Rad Laboratories, USA) instead of DTT.

3.2.7.2 SDS-PAGE

SDS-PAGE was performed using Criterion™ Precast gels in Criterion Dodeca™ cell (Bio-Rad Laboratories, USA) enabling to run 12 gels at a time. IPG strips were carefully placed on top of the gel to ensure optimal contact between them. The running buffer (pH 8.3) was composed of 25 mM Tris, 192 mM glycine and 0.1% (w/v) SDS and was prepared by 10 fold dilution of 10×TGS buffer (Bio-Rad Laboratories, USA). SDS-PAGE was run at the voltage of 200 V for approximately 58 min.

3.2.8 Gel staining, image acquisition and data analysis

Staining was performed using SyproOrange protein gel stain (SIGMA-ALDRICH). Prior to staining, gels were fixed for 1 h in fixing solution composed of 40% ethanol (Altia Oyj, Finland), 2% acetic acid (Merck KGaA, Germany) and 0.0005% SDS (PlusOne, GE Healthcare). After fixation, gels were rinsed twice in MilliQ water and then immersed into staining solution containing 0.02% Sypro Orange stain, 2% acetic acid and 0.0005% SDS (PlusOne, GE Healthcare). The staining was performed for 1 hour followed by rinsing twice with MiliQ water. The gels were then stored in MiliQ water.

The gel images were acquired using AlphaImager HP set at aperture 2, zoom 17.50 and auto focus as well as AlphaView-AlphaImager HP software (ProteinSimple, USA).

Analysis of the gel images was performed using SameSpots software (Totallab, UK). The gel images were aligned by automated calculation of alignment vectors after assigning 40–80 landmark vectors depending on the quality of gels. An ANOVA with p-value ≤ 0.05 was chosen to identify significant changes in spot volume values between what conditions. Another selection criterion for differentially expressed proteins was maximal fold change that was set at ≥ 1.5 .

3.2.9 Spot picking and protein identification

In order to visualize protein spots for identification, re-staining with silver staining method (O'Connell and Stults 1997) was applied. For this purpose, gels were fixed for at least 1 h in fixing solution composed of 30% ethanol (Altia Oyj, Finland), 0.5% acetic acid (Merck KGaA, Germany), at the same time ensuring removal of the Sypro Orange stain, followed by rinsing in 20% ethanol for 10 min and in MilliQ water for 10 min. Next, the gels were sensitized in 0.2% sodium thiosulfate solution (Merck KGaA, Germany) for 1 min and rinsed with MilliQ water for 20 s twice. Staining was applied as holding in 0.2% silver nitrate solution for 30 min followed by rinsing with MilliQ water for 10 s twice. Next, the stain was developed by immersing gels to dedicated solution composed of 3% potassium carbonate (Merck KGaA, Germany) with 50 ml/l sensitization solution (see above) and 700 μ l/l of 37% formaldehyde (Merck KGaA, Germany) typically for 1 to 5 min to achieve the desired intensity. Finally, the staining was terminated by holding in stop solution composed of 5% Trizma®Base (SIGMA-ALDRICH) and 2.5% acetic acid for 1 min. Spots collection was performed by means of 1.5 mm spots picker P2D 1.5 (The Gel Company, USA). Gel specimens containing protein samples were stored at -20°C before further analysis.

Protein specimens were subjected to trypsin digestion to yield peptide mixtures. The identification was conducted in Biocenter (Viikki campus, University of Helsinki). Peptides were then analyzed with liquid chromatography tandem mass spectrometry (LC-MS/MS) using QSTAR Mass Spectrometer (Applied biosystems/MDS Sciex). The data obtained was analyzed with ProteinPilot™ Software (AB SCIEX) and run against NCBI database using MASCOT search engine. In order to obtain the information about proteins several databases were employed including KEGG, Uniprot and BRENDA.

4. RESULTS

4.1 Growth kinetics

The growth rates of *P. freudenreichii* DSM 20271 during logarithmic phase in DMBI+ and DMBI- condition were similar to each other (Fig. 9). Mean generation time values were 4.3 and 4.4 h, respectively. The culture grown in PPA medium without cobalt and DMBI supplementation had generation time of 4.1 h. However, supplementation with cobalt and DMBI enabled to achieve higher cell density. The final OD₆₀₀ measured at 76 h incubation was highest in DMBI+ culture (OD₆₀₀=3.7) as compared to DMBI- (OD₆₀₀=3.46) and PPA (OD₆₀₀= 3.19).

In order to obtain bacterial cells for 2-D electrophoresis at mid-logarithmic growth stage the harvesting time was set at 24 h, when values of the OD₆₀₀ were 0.56-0.91 in DMBI+ and 0.61-0.76 in DMBI- condition.

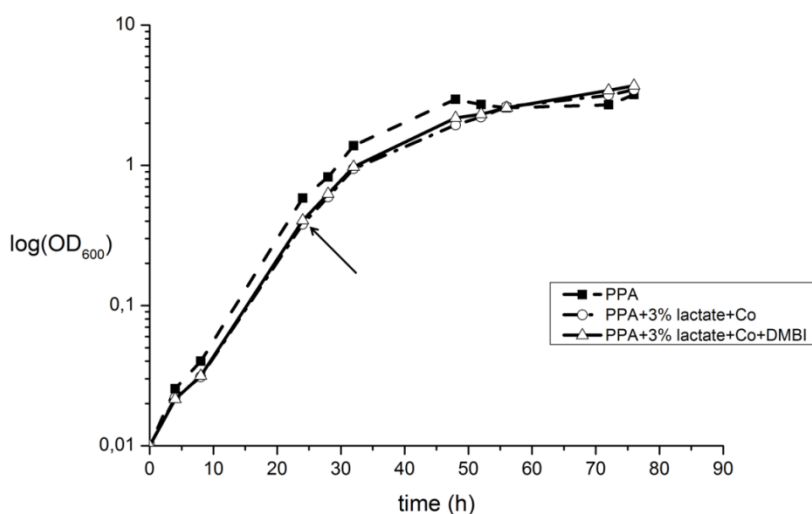


Figure 9. Growth curve of *P. freudenreichii* DSM 20271 cultivated in liquid PPA medium with different supplementations. Harvesting time at mid-logarithmic phase is marked with an arrow

4.2 Vitamin B12 production

Cobalamin levels were determined for DMBI- and DMBI+ condition from three biological replicate cultures. The levels were expressed in ng per cell pellet mass as well as volumetric productivity in ng per ml of media volume. Volumetric productivity reveals the

efficiency of the whole process. The measurements were made at logarithmic growth stage (24 h) and after 168 h incubation. Measurements at 24 h were made for cultures grown in 230 ml PPA media. Determination after 168 h was done for cells harvested from 21 ml culture medium left from harvesting for 2D GE analysis. Three replicates of both DMBI- and DMBI+ cultures were grown under anaerobic condition during all 168 h. Three more replicates of each condition were grown in two step fermentation process, i.e. when after 72 h incubation samples were transferred to aerobic condition for the rest 96 h.

In mid-logarithmic phase at 24 h the level of cobalamin in DMBI+ samples was around twice as high as in DMBI- (15.8 against 30.1 $\mu\text{g/g}$ pellet) (Fig. 10). Volumetric productivity was also higher in DMBI-supplemented samples (0.044 against 0.025 $\mu\text{g/ml}$) (Fig. 11).

By the end of fermentation (168 h) in the absence of oxygen, cobalamin level increased around 8-fold in DMBI- cultures and almost 12-fold in DMBI+ cultures (Fig. 11). The resulting vitamin levels were 0.2 and 0.53 $\mu\text{g/ml}$, respectively. Thus, in DMBI supplemented samples cobalamin level was around 2.7 times as high as in the absence of DMBI. In terms of B12 level in relation to cell mass, the increase is not as dramatic as in case of volume productivity. In DMBI- cultures by the end of fermentation there was slight increase from 15.8 to 17.1 $\mu\text{g/g}$ (Fig. 10). In DMBI-supplemented cultures cobalamin level rose from 30.1 to 38.6 $\mu\text{g/g}$.

In the samples to which two-step process was applied higher cobalamin levels were found both in presence and absence of DMBI. In DMBI- cultures final level of cobalamin was around 51% higher than in the cultures grown anaerobically for 168 h. In case of DMBI+ samples the increment was about 15.5%. Final cobalamin levels in DMBI- and DMBI+ cultures grown in two-step process were 0.3 and 0.6 $\mu\text{g/ml}$, respectively. Thus, the highest cobalamin level was achieved in presence of DMBI combined with two-step fermentation. In terms of B12 level per cell mass in the absence of DMBI, the increment resulting from incubation under aerobic condition for the last 96 h was about 21%. As for DMBI+ samples no significant increase was observed.

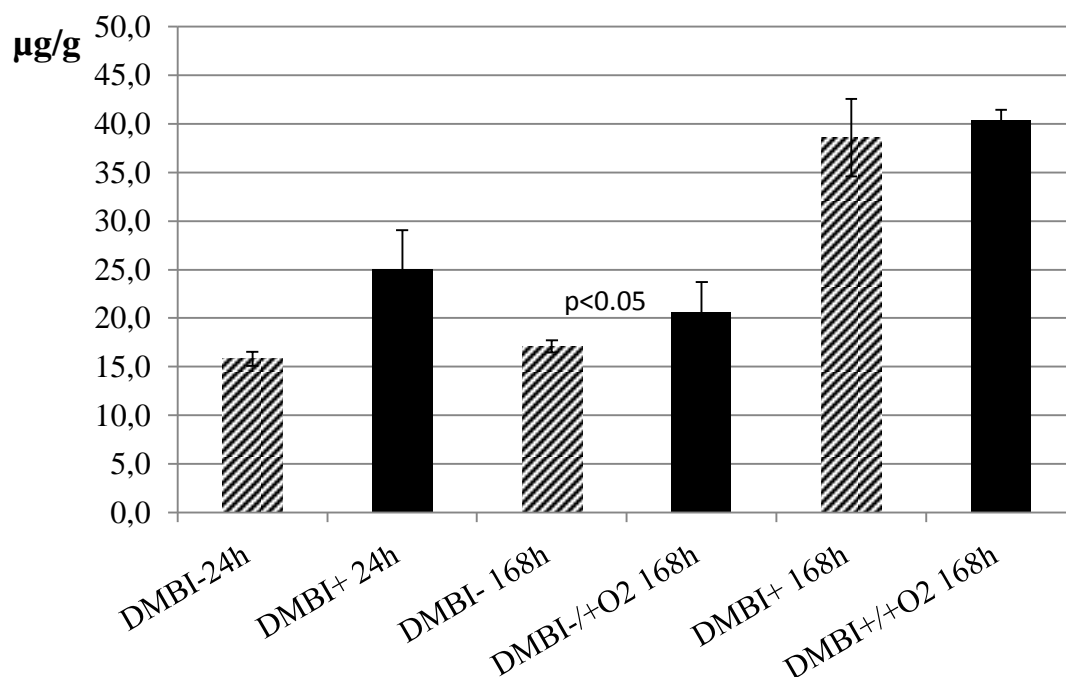


Figure 10. Cobalamin levels produced by *P. freudenreichii* DSM 20271 cultures, µg/g cell pellet. Black bars – DMBI+ condition, Diagonal fill bars – DMBI- condition. DMBI- 168h and DMBI+ 168h were incubated anaerobically. DMBI-/+O2 168h and DMBI-/+O2 168h were grown in two-step process including 72 h anaerobic incubation followed by 96 h aerobic incubation. Error bars show standard deviations.

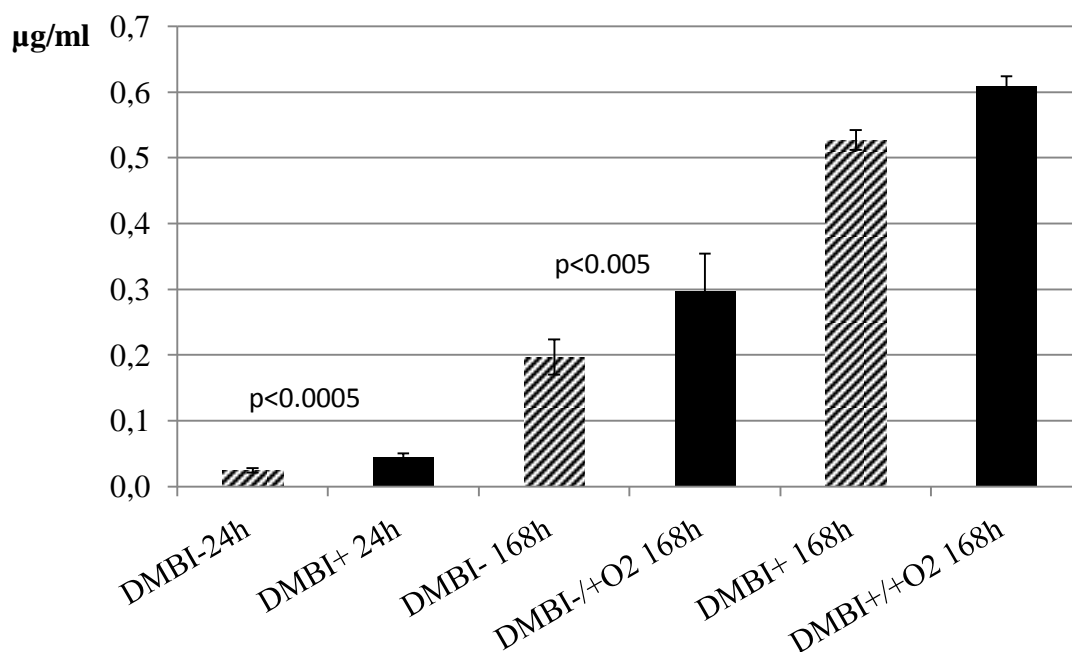


Figure 11. Volumetric production of cobalamin by *P. freudenreichii* DSM 20271 cultures, µg/ml medium. Black bars – DMBI+ condition, diagonal fill bars – DMBI- condition. DMBI- 168h and DMBI+ 168h were incubated anaerobically. DMBI-/+O2 168h and DMBI-/+O2 168h were grown in two-step process including 72 h anaerobic incubation followed by 96 h aerobic incubation. Error bars show standard deviations.

4.3 Proteome maps of *P. freudenreichii* at mid-logarithmic growth stage

Global protein production in *P. freudenreichii* DSM 20271 at the mid-logarithmic growth stage was studied by means of 2D gel electrophoresis. Six biological replicate cultures for each of two conditions (DMBI+ and DMBI-) were used for preparing cell samples. Proteins extracted from these samples were first separated on immobilized pH gradient strips in the pH range from 4 to 7. In the second dimension proteins were separated according to molecular weight in polyacrylamide gels. Proteome maps for DMBI+ and DMBI- conditions were obtained by visualization of gels using Sypro Orange staining. The images were processed using the SameSpots software. At the stage of alignment one replicate of DMBI- condition was excluded, and further analysis was conducted with five replicates for DMBI- and six replicates for DMBI+ condition.

In total, 474 spots were detected. Principle component analysis (PCA) was performed for all spots detected on six gels of DMBI+ and five gels of DMBI- conditions. It showed no significant difference in protein production between these two conditions (Fig. 12).

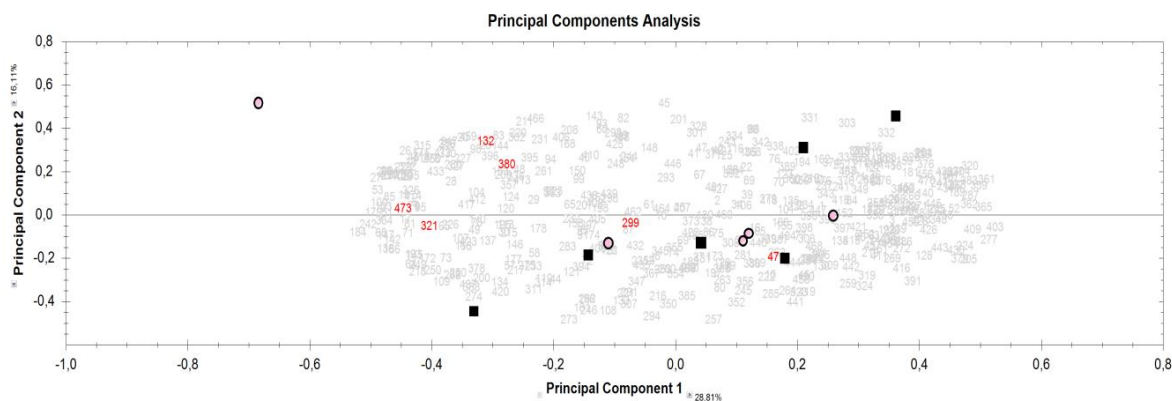


Figure 12. Principle component analysis on spots detected in proteome maps obtained for DMBI- and DMBI+ condition. Individual gels are represented as ● (DMBI-) and ■ (DMBI+)

PCA was also performed only on the spots which had significantly different abundance (ANOVA p-value ≤ 0.05) between condition DMBI- and DMBI+. It showed separation of gels according to the condition (Fig. 13). Principle component 1 accounted for 65.2% of variation between the groups which is much higher than in PCA performed on all the spots (28.8%).

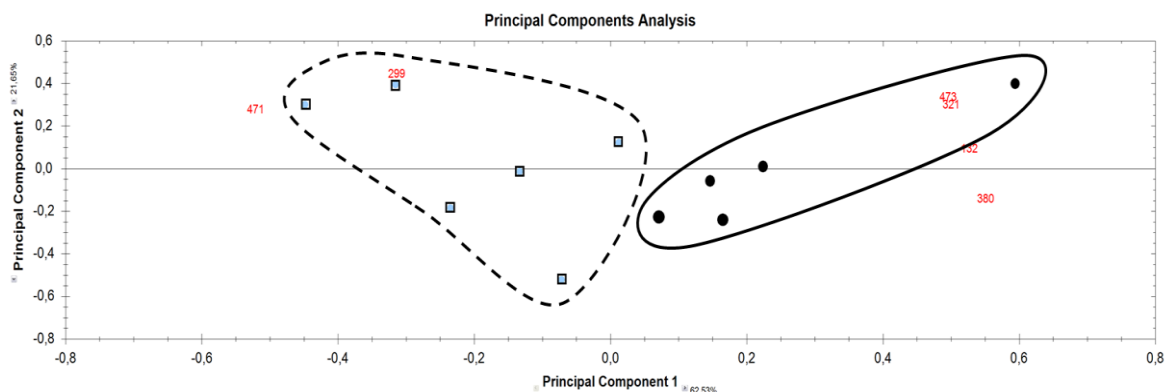


Figure 13. Principle component analysis on spots showing significantly different abundance between conditions DMBI- and DMBI+ (ANOVA p-value ≤ 0.05). Individual gels are represented as ● (DMBI-) and ■ (DMBI+)

Proteome maps of mid-logarithmic *P. freudenreichii* cultures are given in Fig. 14 and 15. Only six spots showed clear differences in relative abundance between DMBI+ and DMBI- condition, i.e. had ANOVA p-value ≤ 0.05 . They were selected for further analysis. Among them the abundance of four spots had maximum fold change ≥ 1.5 . The abundance of spots 5 and 6 had fold change of 1.3 and 1.4, respectively (Table 1), which is less than the selection criteria. Nevertheless, they were selected for further analysis as they had ANOVA p-value below 0.05.

The abundance of only two proteins (corresponding to spots 1 and 5) (Fig. 14) was higher in presence DMBI+ condition (positive fold change values in Table 1). Other four spots (Fig. 15) were significantly more abundant in the absence of DMBI (negative fold change values in Table 1).

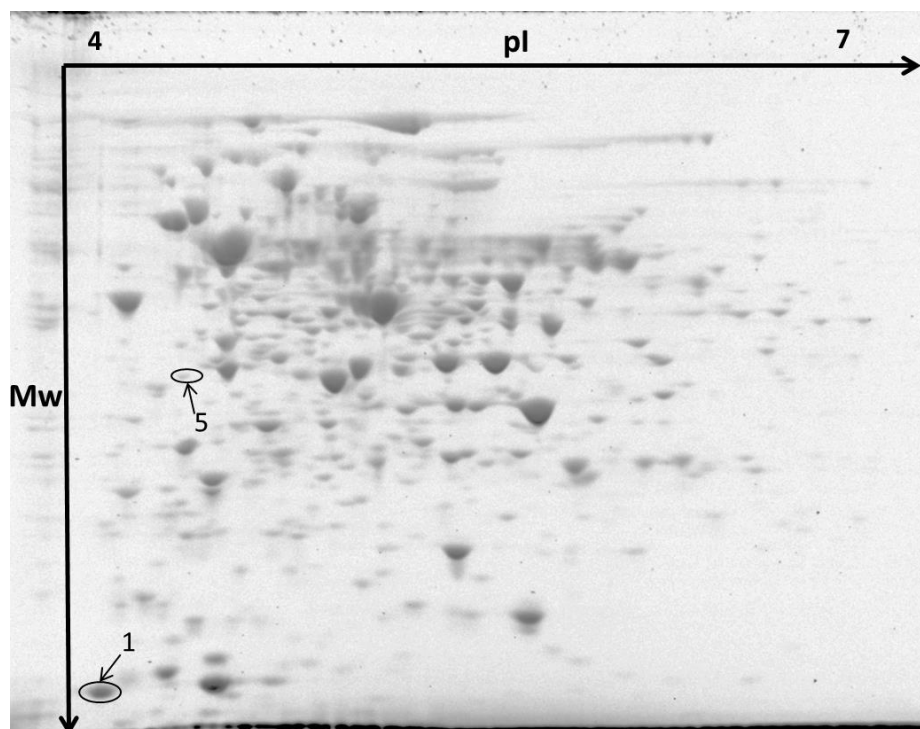


Figure 14. Proteome map of mid-logarithmic culture of *P. freudenreichii* DSM 20271 grown in PPA medium supplemented with 15 ppm DMBI. Spots 1 and 5 outlined and marked with arrows showed higher abundance in DMBI+ condition as compared to DMBI- condition

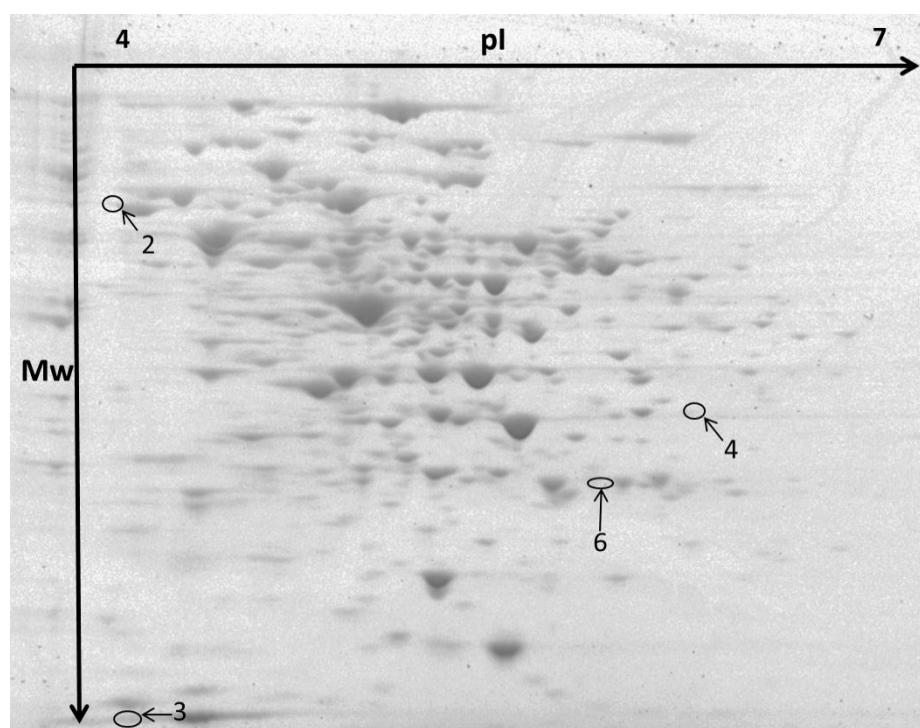


Figure 15. Proteome map of mid-logarithmic culture of *P. freudenreichii* DSM 20271 grown in PPA medium without DMBI supplementation. Spots 2, 3, 4, 6 outlined and marked with arrows showed higher abundance in DMBI- condition as compared to DMBI+ condition

4.4 Identification of six selected proteins spots

All six protein spots which showed differential abundance (ANOVA p-value ≤ 0.05) between DMBI+ and DMBI- conditions were identified and assigned to database entries using KEGG, Uniprot and BRENDA databases. A list of all identified proteins, including accession ID, sequence coverage, Mascot scores, theoretical pI values and molecular weights are shown in Table 1. Several proteins were identified for spots 2, 3, 4, 6 with varying sequence coverage.

Only two protein spots (1 and 5) were more abundant in presence of DMBI. Proteins which could have been induced in this condition included 50S ribosomal protein L7/L12 and carboxylic ester hydrolase identified in spots 1 and 5, respectively.

Other four protein spots (2, 3, 4, 6) were more abundant in DMBI- condition. A group of ribosomal proteins were identified in spots 2 and 3. It is noteworthy that spots 1 (more abundant in DMBI+) and 3 are located close to each other in the same part of gels close to anodic end (Fig. 12 and 13). Most proteins identified for spot 3 are basic with pI values near or above 10. This does not agree with the position on the gel.

Five proteins were identified in spot 4, however, it was cysteine synthase encoded by *cys2* gene which had the however highest Mascot score. Transporter proteins including ABC transporter ATP-binding proteins and branched-chain amino acid ABC transporter ATP-binding protein were among the identifications in spots 2 and 4. In addition, protein involved in lipid metabolism and energy production proteins were identified in these spots as well with lower score and sequence coverage. They included glycerophosphoryl diester phosphodiesterase, anaerobic glycerol-3-phosphate dehydrogenase subunit A.

No exact gene names were found for protein from the spot 6. The protein identified with the highest score was oxidoreductase which could also be a part of lipid metabolism and energy production pathways.

Table 1. Identification of protein spots from proteome maps of mid-logarithmic cultures of *P. freudenreichii* ssp. *freudenreichii* DSM 20271. Spots showed significant differences in abundance in response to presence (DMBI+) and absence of DMBI (DMBI-). Spot numbers refer to Fig. 14 and 15

Spot No	Gene	Description	pI	Mw	Score	Sequence coverage, %	Fold change of DMBI+ relative to DMBI-	ANOVA p-value	Accession number
1	<i>rplL</i>	50S ribosomal protein L7/L12	4.46	13657	1110	89	2.3	0.042	gi 297625768
2	<i>rpsA</i>	30S ribosomal protein S1	4.61	53583	1923	68	-1.6	0.034	gi 297626694
	<i>glpA</i>	anaerobic glycerol-3-phosphate dehydrogenase subunit A	5.58	59534	695	27			gi 297626480
	<i>pyrG</i>	CTP synthase	6.2	64293	455	15			gi 297626545
	PFREUD_07970*	ABC transporter ATP-binding protein	4.9	62791	295	9			gi 297626001
3	<i>rplR</i>	50S ribosomal protein L18	11,4	14014	332	58	-2	0.042	gi 297625798
	<i>rplU</i>	50S ribosomal protein L21	9,73	11235	182	53			gi 297626047
	<i>rpsM</i>	30S ribosomal protein S13	10,73	14018	121	43			gi 297625815
	<i>rplT</i>	50S ribosomal protein L20	10,84	13878	118	26			gi 297626568
	<i>rplN</i>	50S ribosomal protein L14	10,01	13450	100	41			gi 297625792
	<i>rplS</i>	50S ribosomal protein L19	10,36	13247	100	30			gi 297626591
	<i>rsfS</i>	ribosomal silencing factor	4,63	14153	72	24			gi 297626056
	<i>rplL</i>	50S ribosomal protein L7/L12	4,46	13657	55	19			gi 297625768
	<i>rplW</i>	50S ribosomal protein L23	9,64	11519	54	41			gi 297625784
4	<i>cys2</i>	cysteine synthase	5.14	33620	357	30	-1.5	0.017	gi 297626804
	PFREUD_06120*	ABC transporter ATP-binding protein	5.88	30956	309	20			gi 297625365
	<i>ugpQ2</i>	glycerophosphoryl diester phosphodiesterase	4.6	28117	186	16			gi 297627469
	<i>livF</i>	branched-chain amino acid ABC transporter ATP-binding protein	6.19	25206	103	10			gi 297626278
	PFREUD_01850*	dihydroorotate dehydrogenase	4.76	40774	99	12			gi 297625388
5	<i>pf2042</i>	carboxylic ester hydrolase	4.63	35294	449	23	1.3	0.047	gi 297627526
6	PFREUD_18650*	oxidoreductase	5.56	29476	516	26	-1.4	0.046	gi 297627014
	CAD48741*	putative phosphoglycerate mutase	5.86	28351	229	26			gi 76556217

* locus in reference genome for proteins with no detailed information available

5. DISCUSSION

5.1 The effect of DMBI on cell growth

DMBI was added to the cultures at the beginning of incubation. The cell growth in *P. freudenreichii* was not considerably affected by DMBI supplementation as compared to non-supplemented culture. This is in agreement with recent study by Wang et al. (2012) who reported no significant effect of DMBI on growth of *P. freudenreichii* CICC 10019 regardless the time of addition (Wang et al. 2012). However, the earliest time for DMBI addition used in their study was after 24 h of incubation and the level of DMBI was only 0.9 mg/l. By contrast, Marwaha et al. (1983) showed that DMBI infused at the early stages of growth (0 and 48 h) at the level of 15 ppm inhibited the growth of *P. shermanii* 566 and *Propionibacterium* arl AKU, 1251 which resulted in reduced B12 biosynthesis. Since assimilation of lactose from the media in this experiment was not affected, authors speculated that the cultures inhibited by early DMBI addition consumed energy more actively to overcome the constraining action of the compound (Marwaha et al. 1983).

5.2 The effect of exogenous DMBI in growth medium on vitamin B12 biosynthesis

The attachment of lower ligand containing DMBI is the last step of coenzyme B12 (cobalamin) formation. In industrial two-step bioprocess formation of DMBI is facilitated by aeration at the second stage. Aeration is provided for a few days during which endogenous DMBI moiety is formed and linked to cobinamide (Survase et al. 2006). Alternatively, exogenous DMBI is added to the culture media and the process can be conducted anaerobically, i.e. independent from endogenous synthesis of the precursor (Wang et al. 2012).

In available studies, addition of DMBI is normally performed in the stationary phase which restricts possible comparison of our results with other studies. The late DMBI addition strategy was substantiated in the study by Marwaha et al. (1983). Reduced B12 biosynthesis by *P. shermanii* 566 and *Propionibacterium* arl AKU, 1251 was attributed to growth inhibition if DMBI was added at 0-96 h fermentation. The study recommended the infusion of DMBI 24 h before the end of incubation (Marwaha et al. 1983). Cobalamin levels were determined using spectrophotometric technique and expressed in $\mu\text{g/g lactose}\cdot\text{h}^{-1}$ which makes it difficult to compare the results with our cobalamin levels.

In agreement with previous reports, our results indicate that DMBI supplementation considerably enhanced cobalamin biosynthesis. The final level of 0.6 µg/ml obtained in for DMBI-supplemented culture of *P. freudenreichii* DSM20271 in two-step process was lower than that reported by Hugenschmidt et al. (2010) for the same strain which was 0.9 µg/ml. This study employed similar protocol for cultivation and the same level of DMBI. However, in this work DMBI was added 24 h before the end of fermentation (at 144 h). Another difference is that the media used in this screening study was supplemented whey permeate (SWP). Vitamin B12 level was determined using HPLC likewise. This screening study on 37 *P. freudenreichii* strains showed that the species B12 productivity varies in the range 0.0-2.5 µg/ml and on average is higher than that of other species. The highest B12 level of 2.5 µg/ml was reported for strains DF17 and DF28 derived from raw milk (Hugenschmidt et al. 2010).

Another study by Quesanda-Chanto et al. (1998) on *P. freudenreichii* DSM20271 reported considerably higher level of B12 which was 2.7 µg/ml as determined by HPLC method. The strain was cultivated in the medium containing glucose as carbon source at 35°C and controlled pH at 6.5. No supplementation with DMBI was involved, only cobalt at the level of 10 mg/l was added. The level of vitamin B12 was high considering that the fermentation lasted only 72 h strictly under anaerobic condition (Quesada-Chanto et al. 1998).

Ye et al. (1996) also investigated the same strain of *P. freudenreichii* DSM 20271 (under the name IFO 12424) obtained from a different collection. They reported that fully anaerobic fermentation with DMBI added from the very beginning for 68 h yielded 4.64 µg/ml B12 determined spectrophotometrically. This amount is considerably higher than the one obtained in our work (0.53 µg/ml). This may be due to higher levels of DMBI and cobalt which were 70mg/l and 50 mg/l, respectively (Ye et al. 1996).

Recent study by Wang et al. (2012) confirmed the importance of DMBI addition timing. The optimal time for DMBI infusion was corresponding to the highest level of intermediate compound adenosyl-cobinamide. In this study DMBI level was only 0.9 mg/ml and corn steep liquor was used as growth medium. DMBI addition was followed by immediate conversion of cobinamide to cobalamin. Earlier or delayed DMBI infusion resulted in lower cobalamin yield. The highest adenosylcobalamin yield achieved for *P. freudenreichii* CICC 10019 was of about 21 µg/ml when DMBI was added at 84 h

anaerobic incubation at maximum adenosyl-cobinamide level, total incubation time being 120 h (Wang et al. 2012).

Berry and Bullerman (1966) reported higher cobalamin levels determined using microbiological assay in *P. shermanii* ATCC 13973 grown in whey-based medium. At the same level of DMBI (15 ppm) and cobalt (5 ppm) and 168 h anaerobic incubation the final cobalamin level was 5.95 µg/ml. It was considerably higher than in the cultures not supplied with DMBI (2.08 µg/ml). However, at lower DMBI concentration of 10 ppm even higher B12 productivity was achieved (6.35 µg/ml) (Berry and Bullerman 1966). Yongsmith et al. (1982) used immobilized cells of *Propionibacterium* arl AKU 1251 cultivated for five days and DMBI was added at the third day. The final B12 amount produced were about 7 µg/ml and 4.96 µg/ml at DMBI level of 20 mg/l and 10 mg/l, respectively (determined by microbial assay). This was considerably higher than in the absence of DMBI which was about 4.3 µg/ml (Yongsmith et al. 1982).

5.3 The effect of two-step incubation on cobalamin productivity

P. freudenreichii is anaerobic and aerotolerant bacterium which produces cobalamin in high yields only under low level of oxygen (Survase et al. 2006). However, biosynthesis of DMBI requires oxygen. This conditioned the development of two-step fermentation process consisting of anaerobic step followed by shift to aeration for a few more days (Speedie and Hull 1960; Vandamme 1992).

Our results indicate considerable improvement in B12 productivity achieved in two-step process in the absence of DMBI. These results are in agreement with the study by Berry and Bullerman (1966) in which aeration at the rate 215 ml air/(l·min) increased cobalamin production by *P. shermanii* ATCC 13973 from about 2.08 µg/ml to 11.2 µg/ml. Higher aeration rate was not necessary to enhance productivity. However, the highest B12 level (15.04 µg/ml) produced in the absence of DMBI was achieved with high aeration intensity (1000 ml air/(l·min)) and cobalt level of cobalt (15 ppm) (Berry and Bullerman 1966).

In DMBI-supplemented cultures aerobic stage of incubation also increased cobalamin production compared to fully anaerobic fermentation, although to a lesser extent. Even though in our work aeration intensity was not controlled, these results disagree with Berry and Bullerman (1966). Their study indicated that DMBI combined with aeration had

negative effect on B12 production. Hence, no DMBI addition was recommended when low aeration rate was applied (Berry and Bullerman 1966).

Ye et al. (1966), who worked with the same strain *P. freudenreichii* DSM20271, developed cultivation system with periodic variation of dissolved oxygen level which had a great influence on B12 production. It was demonstrated that shift to aerobic condition for a short period (up to 6 h) results in decomposition of propionate. Propionate accumulated in large amounts during anaerobic growth has an inhibitory effect on bacterial cells. Therefore, periodic aerobic incubation enabled higher cell growth. This resulted in increased B12 production. The cell density after 68 h fermentation using periodic system grew as high as 15.02 g/l which was three times as high as in fully anaerobic process. This resulted in production of 9.54 µg/ml B12, which was almost twice as high as when no aeration was provided. DMBI was provided in the medium from the time 0 at higher level than in our work (70 mg/l) (Ye et al. 1996). This indicated that B12 productivity by *P. freudenreichii* DSM20271 can be significantly improved by the combination of DMBI supplementation and aeration.

5.4 Effect of DMBI on protein production

5.4.1 Protein production was not significantly affected by exogenous DMBI in relation to cobalamin biosynthesis

The predicted proteome for *P. freudenreichii* contains 2439 proteins (Falentin et al. 2010). In this work, in total 474 spots were detected using 2D electrophoresis within pH range from 4 to 7. This constitutes less than 20% of the total *P. freudenreichii* proteome. Hence, many proteins including membrane-associated, basic and low-abundant ones were missed out from the analysis. This is one of the shortcomings of 2D gel electrophoresis. The alternative to this can be the technique that overcome the difficulties with protein solubilization, e.g. a GeLC MS/MS method. It involves separation of proteins in polyacrylamide gel followed by in-gel digestion and identification of peptides using nano-LC MS/MS. It enables to maximize the number of identifications in bacterial proteome (Savijoki et al. 2011).

No considerable differences were found in proteome maps between DMBI+ and DMBI- conditions. Differentiation between two conditions could be achieved only based on the abundance of six spots. It is noteworthy that protein production was studied at the mid-

logarithmic growth stage. Cobalamin at this stage was produced in very little amounts. Major part of the vitamin was produced during stationary phase. Cobalamin biosynthetic pathway includes about 30 enzyme-mediated steps (Raux et al. 2000). *P. freudenreichii* produces cobalamin using anaerobic (early cobalt insertion) pathway (Martens et al. 2002). However, it synthesizes DMBI at presence of oxygen (Renz 1998). Aerobic DMBI biosynthesis is known to be catalyzed by BluB enzyme which utilizes riboflavin as a substrate and belongs to a family of ‘flavin destructase’ (Taga et al. 2007). In *P. freudenreichii* *bluB* gene was found to be fused with *cobT* gene encoding the enzyme activating DMBI before the attachment to corrin ring. Production of proteins involved in neither cobalamin nor DMBI biosynthesis was found to be affected in *P. freudenreichii* by exogenous DMBI in the medium during the logarithmic growth stage.

5.4.2 Cysteine synthase may have been induced in the absence of DMBI

The identification with the highest score for spot 4 was cysteine synthase encoded by *cys2* gene. The protein was induced in the absence of DMBI in the growth medium. In total, three cysteine synthases were found in *P. freudenreichii* CIRM-BIA1^T genome according to UniProt database (encoded by *cys1*, *cys2* and *cysK*). The pI value of Cys2 enzyme is 5.14 and that does not agree well with the position of the protein on the gel closer to pH 7 end.

Cysteine synthase is involved in sulfur-containing amino acids metabolism. It transfers sulfhydryl group from hydrogen sulfide to O₃-acetyl-L-serine to yield L-cysteine. Cysteine then can enter transsulfation pathway of methionine biosynthesis. It is converted to methionine in three steps via cystathionine and homocysteine (Rodionov et al. 2004). Methionine participates in the biosynthesis of macroring structure of cobalamin. Corrin ring is formed from all carbons of ALA and methyl carbons of L-methionine (Iida and Kajiwara 2000). Seven methyl groups of corrin ring are derived from methyl groups of L-methionine (Scott et al. 1972).

As it was mentioned before, the abundance of proteins directly involved in cobalamin biosynthesis was not changed by presence or absence of DMBI. However, enhanced cysteine synthesis might have led to active methionine production. Methionine then could be used by bacteria to produce precursor of cobalamin. It is unclear why it was DMBI-condition in which cysteine synthase was expressed to higher extent. As it is seen from cyanocobalamin levels at 24 h, the vitamin production was lower in the absence of DMBI

at this early growth stage. However, only the whole vitamin level was controlled and no investigation was made on the amount of B12 precursors bacteria were producing. Therefore, it is hard to conclude if the cobalamin synthesis was stimulated due to enhanced cysteine synthase production in the cultures growing without exogenous DMBI.

On the other hand, cysteine synthase encoded by *cys2* gene appeared to be induced in *P. freudenreichii* SI41 by bile stress. The enzyme was homologous to cysteine synthase in *B. subtilis* which was known to participate in oxidative stress adaptation (Leverrier et al. 2003). In *P. freudenreichii* cysteine synthase A was induced in response to heat stress. It was suggested to participate in the repair of damaged cells (Anastasiou et al. 2006). In another study, production of cysteine synthase encoded by *cysK* gene was shown to be stimulated by detergent and heat, but not acid, stresses (Vorobjeva et al. 2004).

5.4.3 Ribosomal proteins were induced in both absence and presence of DMBI

Some proteins that appeared to be affected by DMBI belong to the group of ribosomal proteins. In prokaryotes ribosomes consist of two subunits: large (50S) and small (30S) (Aseev and Boni 2011). Ribosomes include from 50 to 80 ribosomal proteins. The interaction between ribosomal proteins and rRNA is essential for optimal ribosome functioning and translation (Wilson and Nierhaus 2005). Apart from their role in translation regulation, some ribosomal proteins were found to exhibit moonlighting properties (Aseev and Boni 2011). Moonlighting is the performance of more than one function by a single protein. The phenomenon was recognized in 1980s and different protein classes were found to exert moonlighting. Additional functions can be fulfilled if proteins are able to use different regions of protein structure, due to post translational modifications and/or differences in binding patterns (Copley 2012).

Most of ribosomal proteins are basic. In *E. coli* ribosomal proteins were separated by 2D-PAGE. Eleven of the 30S and ten of the 50S proteins had pI values ≥ 12.0 , whereas seven of the 30S and fifteen of the 50S proteins had pI values of ≤ 10.0 (Kaltschmidt 1971). The basic nature of most ribosomal proteins is due to their function, i.e. interaction with negative charges of the phosphate residues in rRNA backbone. A few exceptions include proteins S1 and L7/L12 as they interact with other proteins within ribosome (Wilson and Nierhaus 2005).

50S ribosomal protein L7/L12 identified in spot 1 was significantly more abundant in the presence of exogenous DMBI in the medium. It is an acidic protein with pI of 4.46. The spot was located in the left part of the gel accordingly. Unlike other ribosomal proteins L7/L12 is a dimer present in more than one copy in 50S ribosome (Aseev and Boni 2011). L7/L12 interacts with another protein L10 within ribosome forms pentameric complex $L10 \times (L7/L12)_4$ which is stable and can partially resist 6 M urea. L7/L12 forms a characteristic structure of 50S subunit referred to as L7/L12 stalk. L7/L12 is involved in recognition and binding of elongation factors and stimulation of GTPase (Wilson and Nierhaus 2005). In *E. coli* the protein was found to fulfill a moonlighting function of an operon-specific translational repressor. The mechanism of translation inhibition has not been elucidated yet but $L10 \times (L7/L12)_4$ appears to be involved in binding mRNA (Aseev and Boni 2011).

50S ribosomal protein L7/L12 was also among the identifications for the spot 3 located close to spot 1 on the gel. However, this identification had very low score of 55 and sequence coverage of 19% only as compared to 89% for spot 1 identification. This can be the result of the neighboring location of two spots on the gel.

In the absence of DMBI 30S ribosomal protein S1 identified with the highest score in spot 2 was induced. It is an acidic protein with pI value of 4.61 which agrees with the spot position on the gel. S1 is the largest ribosomal protein. It is responsible for binding of mRNA to the 30S subunit at the initial stage of translation (Wilson and Nierhaus 2005). Besides RNA, S1 is also capable of protein-protein interaction. It is the reason for S1 fulfilling numerous functions besides the main one. One of the moonlighting functions of S1 is regulation of translation as it was shown to inhibit the translation of its own mRNA (Aseev and Boni 2011).

Among several ribosomal proteins identified for spot 3 only ribosome recycling protein (encoded by *rsfS* gene) had pI value (4.46) which agreed with the spot location on the gel. In *P. freudenreichii* CIRM-BIA1^T it interacts with ribosomal protein L14 (encoded by *rplN* gene), blocking formation of intersubunit bridge, thus preventing association of the 30S and 50S ribosomal subunits. This results in translation repression (Falentin et al. 2010). RplN protein was also identified in the same spot. However, its pI value of 10.01 disagrees with the spot location on the gel.

Seven ribosomal proteins identified in spot 3 (encoded by *rplR*, *rplU*, *rpsM*, *rplT*, *rplN*, *rplS*, *rplW* genes) that are basic with pI values around 10.0 or higher. It is unclear how these proteins could appear in the acidic region of the gel. Since the spot 3 is localized in the edge of the gel it can be suggested that during IEF basic proteins did not move along the IPG strip from anodic end of IPG strip (pH around 4.0) where the loading was done. In early gel-based separation of *E. coli* ribosomal proteins in the first dimension was achieved using gels with discrete pH values (Kaltschmidt 1971). It can also be suggested that ribosomal proteins were not properly solubilized since they are known to form complexes with each other like in case with L10×(L7/L12)₄ complex which is stable at high urea levels (Wilson and Nierhaus 2005).

Thus, the differential abundance of ribosomal proteins can indicate that translation was affected by DMBI. Also, ribosomal proteins are able to fulfil moonlighting function and they could also be somehow influenced. However, the exact relation between presence of DMBI and ribosomal proteins is not clear.

5.4.4 Carboxylic esterase may have been induced in the presence of DMBI

In the presence of DMBI the abundance of protein spot 5 is the second spot that was higher than in DMBI-. However, the fold change was below 1.5. The only identification for spot 5 was putative carboxylic ester hydrolase encoded by *pf2042* gene. Its pI value of 4.63 agrees with position of the spot on the gel. In *P. freudenreichii* genome, twelve carboxylic esterases were found (Falentin et al. 2010). Pf2042 enzyme was found to be an intracellular esterase (Dherbecourt et al. 2010) but it has not been studied in details. It is involved in lipids metabolism although its exact function is not clear.

5.4.5 In the absence of DMBI ABC transporter ATP-binding proteins may have been induced

Three ABC transporter ATP-binding proteins could have been affected by the absence of DMBI. One of the proteins was branched-chain amino acid ABC transporter ATP-binding protein encoded by *livF* identified in spot 4. Two others were among the identifications in spot 2 and 4. They are involved in transport and binding of proteins and lipoproteins but their exact functions are unclear. In *P. freudenreichii* some ABC transporters were previously reported to be involved in bile stress specific response. It was suggested that they could be a part of efflux system for detoxification (Leverrier et al. 2004). Some ABC

transporters were also found to be induced during heat stress adaptation (Anastasiou et al. 2006).

5.4.6 Lipid metabolism and energy production proteins induced in the absence of DMBI

The abundance of three proteins induced in the absence of DMBI might be related to lipid metabolism and energy production. Glycerophosphoryl diester phosphodiesterase encoded by *ugpQ2* gene was identified in the spot 4. The enzyme catalyzes hydrolysis of glycerophosphodiester yielding respective alcohol and glycerol-3-phosphate (G3P). In *E. coli* the enzyme was shown to have broad substrate specificity and overexpressed in response to phosphate starvation. Under this condition *E. coli* appeared to utilize glycerophosphodiester as a source of phosphate (Ohshima et al. 2008).

The second highest score identification in spot 2 was anaerobic glycerol-3-phosphate dehydrogenase subunit A. The enzyme is encoded by *glpA* gene and was also induced in the absence of DMBI. It is involved in ATP-generating electron transport pathway. It converts G3P into dihydroxyacetone phosphate. In *E. coli* GplA is a part of G3P dehydrogenase operon and was shown to form a dimer GlpAB carrying the catalyzing site for G3P oxidation. The third subunit of the operon, GlpC, accepts reducing equivalents from GlpAB. Oxidoreductase identified with the highest score for spot 6 was found to be homologous to GlpC of *E. coli*. GlpC has iron-sulfur cluster binding property using cysteine rich regions (Cole et al. 1988). According to the Mascot database, oxidoreductase of *P. freudenreichii* also had iron-sulfur binding region. Thus, the three enzymes could constitute successive catalysis of reactions that were involved in glycerophospholipids metabolism and energy generation by the cells grown without exogenous DMBI.

6. CONCLUSIONS

The study evaluated the effect of exogenous DMBI in growth medium on vitamin B12 synthesis and protein production in *P. freudenreichii* ssp. *freudenreichii* DSM 20271. The growth of *P. freudenreichii* was not inhibited by DMBI added to the medium at the beginning of incubation.

DMBI supplementation resulted in 2.7-fold higher level of vitamin B12 compared to the culture grown in absence of DMBI. In addition, two-step process combined with DMBI supplementation enabled the improvement of volumetric productivity of cultures as compared to cultures grown fully anaerobically. This may indicate that in two step process *P. freudenreichii* utilizes exogenous DMBI and produces its own DMBI during aerobic stage for complete cobalamin synthesis simultaneously. On the other hand, aeration could result in decomposition of propionate as an inhibitory agent for *P. freudenreichii* cells.

The highest level of vitamin B12 of 0.6 µg/ml was achieved in two-step process. It is less than in other studies using the same strain but different media, the level and the time of DMBI addition. Hence, the growth conditions for *P. freudenreichii* ssp. *freudenreichii* DSM 20271 can be optimized for better productivity.

Proteome maps were obtained for mid-logarithmic cultures grown in absence and presence of DMBI under anaerobic condition using 2D gel electrophoresis. Less than 20% of total *P. freudenreichii* proteome was displayed on the proteome maps. The 2D GE method may require optimization in order to maximize the number of proteins that can be detected.

The analysis of proteome maps for DMBI- and DMBI+ conditions showed no significant differences in protein production between them. Only six protein spots showed significant differences in abundance. The abundance of proteins directly involved in vitamin B12 biosynthesis was not found to be affected in presence or absence of DMBI. The only indirect evidence of cobalamin biosynthetic pathway to be affected was higher abundance of cysteine synthase in DMBI- as compared to DMBI+ condition. Cysteine synthase could supply cysteine for further conversion to methionine which, in its turn, could be used for corrin ring formation. Possible involvement of cysteine synthase in vitamin B12 synthesis in the absence of DMBI could be investigated further by the analysis of intermediate compounds present before the attachment of DMBI to corring ring.

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